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(57) Abstract

The present invention provides cholesterol modified triple helix forming oligonucleotides and methods for their use. In addition, the present invention provides a method of enhancing sequence specific binding of a synthetic triple helix forming oligonucleotide. The present invention also provides a method of treating a disease comprising administration of a triple helix forming synthetic oligonucleotide modified with a lipophilic compound.

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CHOLESTERYL-MODIFIED TRIPLE-HELIX FORMING OLIGONUCLEOTIDES AND USES THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the field of triple-helix forming oligonucleotides. More specifically, the subject invention relates to cholesteryl-modified triple-helix forming oligonucleotides and methods for their use.

Description of the Related Art.

Triple-helix (triplex) forming oligonucleotides or TFOs are reagents which bind to DNA in a site selective manner. Synthetic single-stranded DNA oligonucleotides targeted to repeating A or G segments within a DNA gene can form stable triplexes at acidic pH. The formation of these triple helices is based upon hydrogen bonding of T to the AT bases in a DNA double helix or duplex and a protonated cytosine (C+) to a GC duplex.

U.S. patent application United States Serial No. 07/453,532 and EPO application No. 90901460.7, published October 9, 1991, disclose an alternative method for making triplex forming oligonucleotides. The

synthetic oligonucleotides bind to a target sequence in duplex DNA forming a collinear triplex by binding to the major groove. For example, triplex forming oligonucleotides may be prepared by scanning the genomic duplex DNA and identifying nucleotide target sequences of greater than about 20 nucleotides having about at least 65% purine bases on one strand; and synthesizing the synthetic oligonucleotide complementary to the identified target sequence, where the synthetic oligonucleotide has a G when the complementary location in the DNA duplex has a GC base pair and has a T when the complementary location of the DNA duplex has an AT base pair.

The triplex strategy has certain advantages, e.g., fewer and less regenerative targets (single copy genes vs. multiple copy mRNAs). When TFOs are targeted to a vital promotor region of a test gene, they have been shown to selectively repress transcription in a dose-dependent manner. Triplexes have been shown to interfere with the function of sequence-specific DNA binding proteins such as restriction enzymes and transcription factors, both *in vitro* and in cultured cells.

Modification of antisense oligonucleotides with lipophilic groups, e.g., cholesterol, increases the uptake of the antisense oligonucleotide by the cell and, subsequently, by the nucleus. However, the uptake of these modified antisense oligonucleotides by the cell occurs in a sequence independent manner. Moreover, cholesterol modification of antisense oligonucleotides produces only non-specific enhancement of biological activity.

There are many problems associated with attempts to chemically modify triplex forming oligonucleotides with lipophilic compounds. First of all, attachment of a lipophilic substance to the oligonucleotide may impede

the synthesis of the triplex forming oligonucleotide and thus reduce the overall quantity of TFO formed. Secondly, triplex forming oligonucleotides are guanosine rich and have a tendency to aggregate in solution. Modification of the triplex forming oligonucleotide with a lipophilic substance such as cholesterol can potentially exacerbate the aggregation problem. Over-aggregation of TFOs would, in turn, decrease binding of the oligonucleotide to its target region. Thirdly, it is not known whether modification of a triplex forming oligonucleotide with a lipophilic compound like cholesterol would interfere with the sequence specific binding that is essential for the TFO's biological effect.

The prior art remains deficient in the absence of a teaching of how to make and use triplex forming oligonucleotides modified with lipophilic compounds so as to significantly enhance the biological actions in a sequence. Moreover, the prior art is deficient in the absence of teachings of efficient methods of attaching cholesterol molecules to TFOs. The present invention overcomes this long felt need and desire in this art.

SUMMARY OF THE INVENTION

An object of the present invention is the provision of a method of potentiating the biological effect of a triplex forming oligonucleotide.

Another object of the present invention is the provision of a method of treating a pathophysiological disease by administering a synthetic triplex forming oligonucleotide having enhanced binding to its target sequence.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention there is provided a method of enhancing a biological action of a synthetic oligonucleotide comprising chemically attaching a lipophilic compound to the oligonucleotide, wherein the oligonucleotide comprises a nucleotide sequence of about at least 20 nucleotides long, said nucleotide sequence including a G and T, is chemically modified with a lipophilic compound and capable of binding to a DNA duplex target to form a triple helix. The method comprises contacting a cell with a synthetic triplex forming oligonucleotide chemically modified with a lipophilic compound.

In accordance with another aspect of the present invention, there is provided a method of treating a pathophysiological disease comprising administration of a synthetic oligonucleotide to an individual. The oligonucleotide is administered in an amount sufficient for cellular uptake and binding to a target sequence. The oligonucleotide comprises a nucleotide sequence of about at least 20 nucleotides long; the nucleotide sequence including G and T and capable of binding to a DNA duplex target to form a triple helix; and a lipophilic compound chemically attached to the nucleotide sequence.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the synthesis of the cholesteryl linker.

Figure 2 shows the synthesis of cholesteryl-CPG and cholesteryl-TentalGel.

Figure 3 illustrates the synthesis of cholesteryl phosphoramidites.

Figure 4 shows the synthesis of thiocholesterol-modified oligonucleotides.

Figure 5 illustrates the calculated intracellular concentration of oligonucleotides in cultured Vero cells. The cells were incubated with control oligonucleotide (triangles) or the oligonucleotide shown in SEQ ID No. 1 (circles). The hollow symbols indicate whole cell concentrations and solid symbols indicate nuclear concentrations.

Figure 6 shows the calculated intracellular concentration of oligonucleotides in cultured U937 cells. The cells were incubated with control oligonucleotide (triangles) or SEQ ID No. 1 (circles). The hollow symbols indicate whole cell concentrations and solid symbols indicate nuclear concentrations.

Figure 7 depicts the calculated intracellular concentration of oligonucleotides in cultured HeLa cells. The cells were incubated with control oligonucleotide (triangles) or SEQ ID No. 1 (circles). The hollow symbols indicate whole cell concentrations and solid symbols indicate nuclear concentrations.

Figure 8 shows the calculated intracellular concentration of oligonucleotides in cultured HeLa cells. The cells were incubated with control oligonucleotide (triangles) or the oligonucleotide shown in SEQ ID No. #3 (circles). The hollow symbols indicate whole cell concentrations and solid symbols indicate nuclear concentrations.

Figure 9 shows the synthesis of cholesteryl supports and phosphoramidite using 6-aminohexanoic acid spacer.

Figure 10. shows the synthesis of cholesteryl supports and phosphoramidite using glycylglycylglycine spacer.

Figure 11 shows the synthesis of phosphoramidite containing longer phosphate linker.

Figure 12 shows the High Performance Liquid Chromatography (HPLC) probe of the synthetic oligonucleotide B-133-55.

Figure 13 shows the labeling and analysis of labeled TFOs on gel.

Figure 14 shows the synthesis of cholesteryl supports and phosphoramidite containing disulfide bond.

Figure 15 shows the synthesis of linker phosphoramidite to prepare cholesteryl oligonucleotide containing phosphate linker.

Figure 16 shows the design and synthesis of cholesteryl oligonucleotide containing oligo-cytidine linker.

DETAILED DESCRIPTION OF THE INVENTION

It is readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

Definitions

As used herein the term lipophilic group or compound means a general class of compounds containing aliphatic, aromatic, alicyclic or heterocyclic groups or combinations thereof. Lipophilic groups may be long hydrocarbon chains molecules. Representative examples of lipophilic compounds include cholesterol, Vitamin E and 1,2-di-O-hexadecyl3-glyceryl.

As used herein the term sequence specific binding means the TFO interacts with the purine strand of a DNA duplex in a Hoogsteen fashion as described by Hogan elsewhere in this specification.

The term "TFO" or "triplex forming oligonucleotide" as used herein refers to the synthetic oligonucleotides of the present invention which are capable of forming a triple helix by binding in the major groove with a duplex DNA structure. The synthetic oligonucleotides bind to a target sequence in duplex DNA forming a collinear triplex by binding to the major groove. For example, triplex forming oligonucleotides may be prepared by scanning the genomic duplex DNA and identifying nucleotide target sequences of greater than about 20 nucleotides having about at least 65% purine bases on one strand; and synthesizing the synthetic oligonucleotide complementary to the identified target sequence, where the synthetic oligonucleotide has a

G when the complementary location in the DNA duplex has a GC base pair and has a T when the complementary location of the DNA duplex has an AT base pair.

As used herein, the term "major groove" refers to one of the grooves along the outer surface of the double-stranded DNA helix which is formed when the sugar-phosphate backbone of the duplex DNA extends further from the axis than the basis. The major groove is important for binding of regulator molecules to specific DNA sequences.

The term "oligonucleotides" as used herein is defined as a molecule comprising two or more de-oxyribe nucleotides or ribonucleotides, preferably more than ten. The exact size depends on many factors, including the specificity and binding affinity.

In an embodiment of the present invention, there is provided a method of enhancing sequence specific binding of a synthetic triplex forming oligonucleotide wherein said oligonucleotide comprises a nucleotide sequence of about at least 20 nucleotides long, said nucleotide sequence including a G & T and is chemically modified with a lipophilic compound and capable of binding to a DNA duplex target to form a triple helix, comprising the step of contacting said oligonucleotide with a cell.

The novel method of the present invention provides for sequence specific binding of modified triplex forming oligonucleotides to a wide variety of DNA duplex target sequences. For example, using the method of the present invention, one with skill in this art may obtain enhanced sequence specific binding of modified triplex forming oligonucleotides to viral genes and oncongenes. Representative examples of viral genes include the immediate

early gene, IE175, of human simplex virus type 2 and the Human Immunodeficiency Virus gene. Representative examples of oncogenes include the c-myc and the c-erb/neu(HER2) gene.

In another embodiment of the present invention, there is provided a method of treating a pathophysiological disease. This method comprises administering a synthetic oligonucleotide to an individual in an amount sufficient for cellular uptake and binding to a target sequence, wherein the oligonucleotide comprises a nucleotide sequence of about at least 20 nucleotides long; the nucleotide sequence including G and T and capable of binding to a DNA duplex target to form a triple helix; and a lipophilic compound chemically attached to the nucleotide sequence.

Attachment of cholesterol to TFOs increases the TFO's uptake and cellular efficacy. However, preparation of TFOs containing cholesterol is challenging both in synthesis and purifications. Due to aggregation problems, the solubility of cholesteryl modified TFOs in water or aqueous buffers has been low. In large scale purification of 30-35 bases long TFOs (to obtain 500 mg to a gram quantity of final product) it is often observed that passage of these modified TFOs through 10,000 to 100,000 molecular weight cut off size membranes has been difficult. This is attributed to the aggregation and probably micellar formation of cholesteryl modified TFOs under high concentrations TFOs and salts. TFOs without cholesteryl modifications pass through these membranes easily. Because of low solubility ($\geq$ 2 to 5 mg/ml) in water, cholesteryl modified TFOs in cell culture media often remain as an "oil overlay" due to poor partitioning of these compounds into the aqueous layer presenting problems in antiviral evaluation. Even

under these conditions, the uptake and cellular efficacy properties of these compounds are enhanced in comparison with non-cholesteryl modified TFOs.

Generally, the novel synthetic oligonucleotides of the present invention may be used to treat a wide variety of pathophysiological diseases. Preferably, the synthetic oligonucleotides of the subject invention may be used to treat cell proliferative states, for example, cancer and viral infections, e.g., herpes simplex virus and the human immunodeficiency virus. Thus, the novel methods of the present invention may be used to treat, inter alia, breast, lung, and cervical cancer.

In one embodiment of the compositions of the present invention, the synthetic TFOs may have the sequence TFO-Linker-cholesterol. Generally, the lipophilic compound may be any that allows for and enhanced cellular uptake and biological effect of the synthetic oligonucleotide. Preferably, the lipophilic compound is selected from the group consisting of cholesterol, Vitamin E, 1,2-di-O-hexadecyl-3-glyceryl, and generally compounds having long hydrocarbon chains. In addition, the lipophilic compounds may generally be molecules that contain aliphatic, aromatic, alicyclic or heterocyclic groups or combinations thereof.

The lipophilic compound may be chemically attached to the nucleotide sequence by using a linking compound. Preferably, the linking compound is selected from the group consisting of 2-cyanoethyl solketal, 3-aminopropyl solketal, N-((Cholesteryloxy)carbonyl)-3-aminopropyl solketal, cholesterol-CPG, cholesterol-TentaGel, 2-cyanoethyl-N,N-diisopropyl 1-O-(4A'-dimethoxytrityl)-3-O-(N-(cholesteryl-oxy)carbonyl-3-aminopropyl)-glyceryl

phosphoramidite and thiocholesterol compounds as described in Example 12. In addition, other linking compounds are exemplified in Figure 9 by compounds 20, 22, 23, and 24; in Figure 10 by compounds 27, 29, 30 and 31; in Figure 14 by compounds 47-50 and in Figure 15 by compounds 53-56.

In another embodiment of the present invention, the synthetic TFOs may have the sequence TFO-X-Linker-cholesterol, wherein X is either a multi-phosphate group or an oligo-cytidine group. TFOs containing multiple linker phosphates (preferably 1-6 phosphates) and cholesterol were prepared to enhance the solubility of the TFO and are more likely to be subjected to cleavage by phosphodiesterase inside the cell thus releasing the cholesterol and TFOs free. Representative examples of TFOs containing a multi-phosphate group are 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl(glycerol)-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)-6-aminohexanoyl)aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)glycylglycylglycyl)-3-aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N(3'-N(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanoyl)aminopropaneglycol, and 1-O-(4,4',dimethoxytrityl)-2-N(2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycol.

Similarly, TFOs containing an oligocytidine sequence (preferably 4-8 Cs) inserted between the TFO and the linker containing the cholesterol were prepared. A small stretch of Cs were susceptible to endonuclease. By inserting such a sequence, the TFO would be cleaved at Cs more readily inside the cell and the TFO can be released from the cholesterol. Deoxy or ribo Cs can be used. A person having ordinary skill in this art would readily

recognize that one may use sequences other than multi-phosphate or oligo-cytidine group to facilitate the release of the TFO within the cell. Representative examples of TFOs containing an oligo-cytidine group is selected from the group consisting of 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glycerol-2-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)-6-aminohexanoyl)aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)glycylglycylglycyl)-3-aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N(3'-(N-(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanyol)aminopropaneglycol, and 1-O-(4,4'-dimethoxytrityl)-2-N(2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycol.

The lipophilic compound may be chemically attached to the either the 3' or the 5' end of the nucleotide sequence of the synthetic oligonucleotide. Preferably, the lipophilic compound is chemically attached to the 3' end of the nucleotide sequence. The triplex forming oligonucleotides useful in the methods of the present invention may be administered to any individual. An individual is defined as a human or any animal. Accordingly, the present invention also encompasses pharmaceutical compositions containing the novel triplex forming oligonucleotides of the present invention. Such pharmaceutical compositions would also suitably contain a form of pharmaceutically acceptable vehicle, e.g., an oil base vehicle, water or saline. These pharmaceutical compositions of the present invention may be administered in a variety of forms and administration routes well-known to those with skill in this art.

The following examples are provided as illustrations of the present invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Synthesis of linker containing cholesterol

(Scheme 1)

Synthesis of 2-cyanoethyl solketal

Solketal (26.4 g, 200 mmole, Aldrich Chem. Co.) and acrylonitrile (21.28 g, 400 mmole, Aldrich Chem. Co.) were dissolved in anhydrous THF (500 ml, Aldrich Chem Co.) in a three necked round bottom flask (1000 ml). The mixture was cooled in an ice cold water bath under argon atmosphere. To the stirred solution, sodium hydride (0.96 g, 40 mmole, Aldrich Chem. Co.) was added slowly. After the addition, the mixture was allowed to warm to room temperature and was stirred for additional 1 hour. Then, 100 ml of water was added dropwise to give a bright yellow suspension which was concentrated to 50 ml to remove THF. Subsequently, 200 ml of water was added to the residue. The resulting mixture was filtered to separate yellow solid, and the filtrate was extracted with dichloromethane (200 ml, 3 times). The combined solvent extract was washed with saturated $NaHCO_3$ aqueous solution (200 ml, twice), brine (200 ml, twice) and the organic phase was dried over anhydrous Na_2SO_4 and concentrated to give a brown oil (45 g), which was distilled under reduced pressure to provide a product. (29.46 g, yield : 79.5%) as a colorless liquid (bp .122-3°C). ^{1}HNMR ($CDCl_3$): ∂ 1.35 and 1.42(s,s, $CCH_3)_2$, 6H), 2.71 (t, J=6.3 Hz, 2H, $C\underline{H}_2CN$), 3.50-3.59 (m,2H), 3.69-

3.76 (m, 3H), 4.05 (dd, J=8.2 Hz, J=6.4 Hz, 1H), 4.24 (quintet, J=5.5 Hz, 1H, C2-CI:i-O-).

EXAMPLE 2

Synthesis of 3-aminopropyl solketal

2-Cyanoethyl solketal (29.46 g, 159 mmole) and cobalt (II) chloride.6H_20 (76.1 g, 320 mmole, Aldrich Chem. Co.)were dissolved in 900 ml of MeOH in a three necked-round bottom flask (2000 ml). The mixture was stirred in an ice cold bath. To the mixture was added slowly sodium borohydride (60.16 g, 1.59 mole, Aldrich Chem. Co.). After the addition, the mixture was stirred for 1 hour at room temperature and then 320 ml of concentrated ammonia solution was added. The resulting mixture was filtered and the filtrate was concentrated to 250 ml to remove methanol. The aqueous layer was extracted with dichloromethane (250 ml, 3 times). The combined solvent extracts was washed with brine (200 ml, 3 times), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give a brown oil (30 g) which was distilled on high vacuum pump to yield 16.0g of product (yield: 60%) as a colorless liquid (bp. 89-90° C). ^1H NMR ($CDCl_3$): ∂ 1.36 and 1.42(s,s, $C(CH_3)_2$, 6H),1.72(quintet J=6.5 Hz,C-CH_2-C , 2H), 2.79 (t, J=6.8 Hz, 2H, C$\underline{H}_2$-NH2), 3.41-3.58 (m, 2(C$\underline{H}_2$O), 4H), 3.72 (dd, J=6.64 Hz, J=6.60 Hz, H, $CH_2N\underline{H}$), 4.04 (dd, J=6.56, J=6.50, H, $CH_2N\underline{H}$), 4.25 (quintet, J=5.9 Hz, H, C_2-C$\underline{H}$-O).

EXAMPLE 3

Synthesis of N – ((Cholesteryloxy)carbonyl)-3-aminopropyl solketal

3-Aminopropyl solketal (5.0 g, 26.4 mmole) was dissolved in anhydrous pyridine (50 ml, Aldrich Chem. Co.), the solution was cooled in an ice cold water bath. To the stirred solution was added cholesterol chloroformate (11.87 g, 26.4 mmole, Aldrich Chem. Co.). The reaction mixture was stirred for 15 minutes at cold temperature and 3 hours at room temperature. The resulting reaction mixture was concentrated to a heavy oil to remove pyridine. The oil was re-dissolved in dichloromethane (250 ml), washed with saturated aqueous sodium bicarbonate solution (200 ml twice), brine (200 ml, twice) and the organic phase was dried over anhydrous Na_2SO_4. After removal of solvent, the crude oil was purified by silica gel column chromatography using a gradient of CH_2Cl_2 and CH_2Cl_2: MeOH 19:1, as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide product [e] (13.98 g, yield: 85%) as a light yellow solid foam. ^1H NMR ($CDCl_3$): ∂ 0.68 (s, 3H, -C<u>H</u>$_3$, chol.), 0.86 (d, J=6.8 Hz, 3H, -C<u>H</u>$_3$, chol.), 0.87 (d, J=6.8 Hz, 3H, -C<u>H</u>$_3$, chol.), 0.91 (d, J=6.68 Hz, 3H, -C<u>H</u>$_3$, chol.), 0.99 (s, 3H,-C<u>H</u>$_3$, chol.), 1.36 and 1.43(s,s, C(C<u>H</u>$_3$)$_2$, 6H), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08 Hz, 2H, C-C<u>H</u>$_2$-C, linker), 3.23-3.16 (m, 4H, 2(C<u>H</u>$_2$), linker), 3.53-3.46 (m, 4H, 2(C<u>H</u>$_2$), linker), 3.95 (m, H, C2=C<u>H</u>-O-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4.72, H, C<u>H</u>=C-, chol.).

EXAMPLE 4

Synthesis of 1-O-(4,4' dimethoxytrityl)-3-O-(N-(cholesteryloxy) carbonyl-3-aminopropyl) glycerol

N-((Cholesteryloxy)carbonyl)-3-aminopropyl solketal (3.1 g, 5.15 mmole) was dissolved in 1N HCl (10 ml), methanol (10 ml) and dichloromethane (10 ml). The mixture was stirred at room temperature for 1 hour. The reaction mixture was concentrated to dryness and co-evaporated with anhydrous pyridine (25 ml, 3 times) to provide a light yellow oil which was re-dissolved in pyridine (30 ml). To the solution was added 4,4'-dimethoxytrityl chloride (2.09 g, 6.18 mmole, Aldrich Chem. Co.). The solution was stirred at room temperature for 1 hour and then methanol (10 ml) was added. The resulting mixture was concentrated to dryness to remove pyridine and methanol and re-dissolved in dichloromethane (200 ml) which was washed with saturated $NaHCO_3$ aqueous solution (100 ml, twice), brine (100 ml, twice). The organic phase was dried over anhydrous Na_2SO_4 and concentrated to give a light yellow oil (4.7 g). The crude oil was purified by silica gel column chromatography using a gradient of CH_2Cl_2 : EtOAc 1: 1 and CH_2Cl_2, as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide product 7 (2.96 g, yield: 66.4%) as a light yellow solid foam. ^1H NMR ($CDCl_3$): ∂ 0.68 (s, 3H, -C$\underline{H}_3$, chol.), 0.86 (s, 3H, -C$\underline{H}_3$, chol.), 0.87 (d, J=1.9 Hz, 3H, -C$\underline{H}_3$, chol.), 0.91 (d, J=6.68 Hz, 3H, -C$\underline{H}_3$, chol.), 0.99 (s, 3H,-C$\underline{H}_3$, chol.), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08 Hz, 2H, C-C$\underline{H}_2$-C, linker), 3.23-3.16 (m, 4H, 2(C$\underline{H}_2$), linker), 3.53-3.46 (m, 4H, 2(-C$\underline{H}_2$), linker), 3.78 (s, 6H, 2(O-C$\underline{H}_3$),

DMT), 3.95 (m, H, C2=CH-O-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4.72, H, CH=C-, chol.), 7.42-6.80 (m, 13H, DMT).

EXAMPLE 5

Synthesis of 1-O-(4, 4'-dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryl oxy)carbonyl-3 aminopropyl)glycerol

1-0-(4,4'-Dimethoxytrityl)-3-0-(N-(cholesteryloxy)carbonyl-3-aminopropyl)glycerol (~, 2.96 g, 3.42 mmole) was dissolved in anhydrous pyridine. To the solution was added succinic anhydride (0.51 g, 5.13 mmole, Fluka Chem. Co.), 4-dimethyl aminopyridine (0.33 g, 2.7 mmole, Aldrich Chem. Co.). The mixture was stirred for 2 hours. The reaction mixture was concentrated to an oil to remove pyridine. The oil was re-dissolved in dichloromethane (200 ml), washed with 10% aqueous citric acid solution (150 ml, twice) and water (150 ml, twice). The organic phase was dried over anhydrous Na_2SO_4. After concentration, the crude oil was purified by silica gel column chromatography using a gradient of CH_2Cl_2 and CH_2Cl_2 : MeOH 19:1, as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide the product (2.34 g, yield: 70%) as a white solid foam. ^1H NMR ($CDCl_3$): ∂ 0.68 (s, 3H, -CH_3, chol.), 0.86 (d, J=6.8 Hz, 3H, -CH_3, chol.), 0.87 (d, J=6.8 Hz, 3H, -CH_3, chol.), 0.91 (d, J=6.68 Hz, 3H, -CH_3, chol.), 0.99 (s, 3H,-CH_3, chol.), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08 Hz, 2H, C-CH_2-C, linker), 2.68 (br s, 4H,

2(CH_2), succinyl), 3.23-3.16 (m, 4H, 2($C\underline{H}_2$), linker), 3.53-3.46 (m, 4H, 2(-$C\underline{H}_2$), linker), 3.78 (s, 6H, 2(0-$C\underline{H}_3$), DMT), 3.95 (m, H, C_2--$C\underline{H}$-O-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4.72, H, $C\underline{H}$=C-, chol.), 7.42-6.80 (m, 13H, DMT).

EXAMPLE 6

Synthesis of Cholesterol-CPG Support

(Scheme 2)

Procedure 1:

1-0-(4,4'-Dimethoxytrityl)-2-O-succinate-3-0-(N-(cholesteryl-oxy)carbonyl-3-aminopropyl)glycerol (0.49 g, 0.5 mmole) was dissolved in anhydrous dioxane (8 ml, Aldrich Chem. Co.) containing 0.4 ml of pyridine. To the solution was added p-nitrophenol (60 mg, 0.5 mmole, Aldrich Chem. Co.), and 1,3-dicyclohexylcarbodiimide (200 mg, 1 mmole, Aldrich Chem. Co.). After stirring for 1 hour, the reaction mixture was filtered to separate N,N'dicyclohexylurea and the filtrate was added to aminopropyl CPG (1 g, loading capacity : 37 μmole/g) in anhydrous DMF (10 ml, Aldrich Chem. Co.). Triethylamine (2 ml) was added to the reaction mixture which was swirled slowly overnight at room temperature. Capping reagents A and B (15 ml each, MilliGen Biosearch Inc.) were then added to the reaction mixture. The mixture was stirred for 30 minutes to cap the unreacted free amino groups on support. Cholesterol-CPG support was collected by filtration, washed with DMF (5 ml, 3 times), methanol (5 ml, 3 times), and ether (5 ml, 3 times), and dried on a high vacuum pump overnight. Cholesterol loading was estimated

by the measurement of trityl cation released by acidic treatment of synthesized support, and was estimated to be 18 μmole/g.

Procedure 2:

1-O-(4,4'-Dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryl-oxy) carbonyl-3-aminopropyl) glycerol (1.62 g, 1 .65 mmole), O-benzotriazole-1-yl-N,N,N',N'-tetramethyluronium-tetrafluoroborate (0.53 g, 1.65 mmole, Fluka Chem. Co.), 1-hydroxy benzotriazole hydrate (0.22 g, 1.65 mmole, Fluka Chem. Co.), N-ethyl morpholine (0.19 g, 1.65 mmole, Fluka Chem. Co.) were dissolved in DMF anhydrous (25 ml). After 5 minutes of shaking, to the mixture was added aminopropyl CPG (3 g, loading capacity : 37 μmole/g). The reaction mixture was swirled slowly for 3 hours. Capping and washing steps were done as described above. The loading of cholesterol was estimated to be 33 μmole/g.

EXAMPLE 7

Synthesis of Cholesterol-TentaGel Support

(Scheme 2)

1-O-(4,4'-Dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryloxy) carbonyl-3-aminopropyl) glycerol (3.2 g , 3.26 mmole), O-benzotriazole-1-yl-N,N,N',N'-tetramethyluronium-tetrafluoroborate (1.05 g, 3.26 mmole , Fluka Chem. Co.), 1-hydroxy benzotriazole hydrate (0.44 g, 3.26 mmole , Fluka Chem. Co.), N-ethyl morpholine (0.37 g, 3.26 mmole, Fluka Chem. Co.) were dissolved in anhydrous DMF (76 ml). After 5 minutes of shaking, to the mixture was added tentagel-NH_2 (10 g, loading capacity: 220 μmole/g). The

reaction mixture was swirled slowly for 3 hours. Capping and washing steps were done as described above. The loading of cholesterol on tentagel support was estimated to be 152 μmole/gram. Loading of cholesterol onto TentaGel support using p-nitrophenol and DCC yielded only 22 μmole/g of support.

EXAMPLE 8

Synthesis of 2-cyanoethyl-N, N-diisopropyl 1-O-(4A'-dimethoxytrityl)-3-O-(N-(cholesteryl-oxy)carbonyl-3-aminopropyl)-glyceryl phosphoramidite (Scheme 3)

1-O-(4,4' -Dimethoxytrityl)-3-O-(N-(cholesteryloxy)carbonyl-3-aminopropyl) glycerol (1.5 g, 1.74 mmole) was dissolved in distilled dichloromethane (15 ml). To the solution was added distilled N,N'diisopropylethylamine (0.45 g, 3.48 mmole) and 2-cyanoethyl-N,N'-diisopropylaminochlorophosphine (0.45 g, 1.9 mmole, Aldrich Chem. Co.). The mixture was stirred for 1 hour at room temperature, under argon atmosphere. After stirring, the reaction mixture was diluted with 50 ml of CH_2Cl_2 and washed with 5% $NaHC0_3$ aqueous solution (50 ml, twice), brine (50 ml, twice), the organic phase was dried over anhydrous $MgSO_4$ and concentrated to give a light yellow oil (2.3 g). The crude oil was purified on a silica gel column using ethyl acetate:dichloromethane:triethylamine (49:49:2) solution as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide a product (1.3 g, yield: 70%) as a white solid foam. ^{31}P NMR ($CDCl_3$): ∂ 149.82 and 149.69; ^1H NMR ($CDCl_3$): ∂ 0.67 (s, 3H, -C$\underline{H}_3$, chol.), 0.85 (s, 3H, -C$\underline{H}_3$, chol.), 0.87 (s, 3H, -C$\underline{H}_3$, chol.), 0.91 (d, J=6.68 Hz, 3H, -C$\underline{H}_3$, chol.), 1.0 (s, 3H,-C$\underline{H}_3$, chol.), 1.14-1.20

(m, 12H, diisopropyl), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08 Hz, 2H, C-C$\underline{H}_2$-C, linker), 2.6(m, 2H, CH_2-0-P), 3.23-3.66 (m, 6H, 2(C$\underline{H}_2$), linker, CH_2CN), 3.53-3.46 (m, 4H, 2(-C$\underline{H}_2$), linker), 3.78 (s, 6H, 2(0-C$\underline{H}_3$), DMT), 3.95 (m, H, C2--C$\underline{H}$-0-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4-.72, H, CHC-, chol.), 7.42-6.80 (m, 13H, DMT).

EXAMPLE 9

Synthesis of 2(N-(Cholesteryloxy) carbonyl-2-aminoethoxy) ethanol (Scheme 3)

2-(2-Aminoethoxy) ethanol (1 g, 9.5 mmole, Aldrich Chem. Co.) was dissolved in anhydrous pyridine (5 ml) and dichioromethane (10 ml). Cholesterol chloroformate (5, 4.3 g, 9.5 mmole) was added slowly to the mixture and stirred for 1 hour at room temperature. After stirring, the reaction solution was concentrated to dryness under reduced pressure to remove pyridine. The crude oil was re-dissolved in dichloromethane (250 ml), washed with saturated aqueous sodium bicarbonate solution (200 ml twice), brine (200 ml, twice) and the organic phase was dried over anhydrous Na_2S0_4. After concentration, the resulting crude material was purified by a silica gel column chromatography using a gradient of CH_2Cl_2 and CH_2CI_2: Me0H 19:1, as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide product (3.71 g, yield: 76%) as a white solid foam. ^{1}H NMR ($CDCl_3$): ∂ 0.68 (s, 3H, -C$\underline{H}_3$, chol.), 0.86 (d, p=6.64 Hz, 3H, chol.), 0.87 (d, J=6.64, 3H, -C$\underline{H}_3$), 0.91 (d, J=6.56 Hz, 3H, -C$\underline{H}_3$, chol.), 1.01 (s, 3H,-C$\underline{H}_3$, chol.), 2.59-0.95 (m, 29H, cholesterol), 3.37 (quartet, J=5.16,

2H, -CH_2-0, linker), 3.57 (m, 4H, -CH_2-O-CH_2-, linker), 3.73 (t, J=4.92 Hz, 2H, -CH_2-NH, linker), 4.50 (m, H, chol.), 5.36 (d, J=4.72, H, CH=C-, chol.).

EXAMPLE 10

Synthesis of 2(N-(Cholesteryloxy)carbonyl-2-aminoethoxy) ethyl-2-Cyanoethyl--N,N-diisopropylphosphoramidite

(Scheme 3)

2(N-(Cholesteryloxy) carbonyl-2-aminoethoxy) ethanol (3 g, 5.8 mmole) was dissolved in distilled dichloromethane (60 ml). To the solution was added distilled N,N'-diisopropylethylamine (1.5 g, 11.6 mmole) and 2-cyanoethyl-N,N'-diisopropylaminochlorophosphine (1 .64 g, 6.9 mmole, Aldrich Chem. Co.). The mixture was stirred for 1 hour at room temperature, under argon atmosphere. After stirring, the reaction mixture was diluted with 200 ml of CH_2Cl_2 and washed with 5% $NaHC0_3$ aqueous solution (50 ml, twice), brine (50 ml, twice), the organic phase was dried over anhydrous $MgS0_4$ and concentrated to give a light yellow oil which was re-dissolved in acetonitrile (6O ml) and extracted with hexane (100 ml x 5 times). The combined hexane layers was concentrated under reduced pressure to give a product (2.5 g, yield: 60%) as a white solid foam. ^{31}P NMR ($CDCl_3$): ∂ 149.28 ^1H NMR ($CDCl_3$): ∂ 0.68 (s, 3H, -CH_3, chol.), 0.85 (s, 3H, -CH_3, chol.), 0.87 (s, 3H, -CH_3, chol.), 0.91 (d, J=6.44 Hz, 3H, -CH_3, chol.), 1.0 (s, 3H,-CH_3, chol.), 2.59-0.95 (m, 29H, cholesterol), 1.20 (m, 12H, 4 (CH_3), linker), 1.73 (quintet, J=6.08 Hz, 2H, C-CH_2-CH_2C, linker), 2.64(t, J=6.4, 2H, CH_2-0-P) 3.23-3.16 (m, 4H, 2(CH2), linker), 3.63-3.56 (m, 4H, 2(-CH_2), linker), 3.90-365 (m, 2H,

H-C(CH_3), 3.81 (m, H, C2--C<u>H</u>-0-, linker), 4.49 (m, H, chol.), 5.37 (d, J=4.72, H, C<u>H</u>=C-, chol.).

EXAMPLE 11

Synthesis of cholesteryl modified oligonucleotides

0.2 μmole to 35 μmole scale syntheses were carried out for the synthesis of TFO's. Appropriate quantity of the support was used depending on the scale.The oligonucleotides were synthesized on Applied Biosystems Models 380B , 394Z and or Milligen Models 8700 and 8800. Phosphoramidite chemistry was used. Deblocking was carried out under standard conditions.

Crude oligonucleotides were purified on a Pharmacia FPLC system by anion exchange chromatography on a Q- Sepharose column (1 cm x 10 cm). The crude oligonucleotide was cleaved from CPG using approximately 2 ml of concentrated NH_4OH at 56° C overnight. After filtering off CPG, the NH_4OH solution was then pumped on to the Q-HP column, which has been equilibrated with 25% buffer B. The elution buffers were: 0.5 M NaCl with 10 mM NaOH (Buffer A) and 1.5 M NaCl with 10 mM NaOH (Buffer B). The flow rate was 5 ml/min. The gradient was 75% buffer A and 25% buffer B for 10 minutes. Then the gradient was changed to 40% buffer A and 60% buffer B for 50 minutes. Finally, the column was washed with 100% buffer B for 20 minutes followed by equilibration with buffer A for 10 minutes. Unmodified full length oligonucleotide (38 mers) were generally eluted with 42% buffer B. Cholesteryl modified full length oligonucleotide was eluted with 54% buffer. The oligonucleotides were desalted on an Amicon TCF cell containing a 10K cut off size membrane filter by passing 5

liters of MilliQ water through the unit. The concentrated and desalted oligonucleotide was then removed from the amicon unit and lyophilized. Typically, the isolated yield was about 30% for unmodified oligonucleotides and about 20-25% for cholesteryl modified oligonucleotides.

The following sequences were synthesized with cholesterol.

Control for SEQ ID No. 1:

5' -TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGGTGNH2 -3'

SEQ ID No. 1:

5' -TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGGTG-Chol. -3'

Control for SEQ ID No. 2:

5' - GTGGTGGTGGTGTTGGTGGTGGTTTGGGGGGTGGGG-NH_2 -3'

SEQ ID No. 2:

5' - GTGGTGGTGGTGTTGGTGGTGGTTTGGGGGGTGGGG-Chol. -3'

Control for SEQ ID No. 3:

5' -TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-NH2 -3'

SEQ ID No. 3:

5' -TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-Chol. -3'

Control for SEQ ID No. 4:

5' -GTGTGTTGTGGGGGGTGGGGGTGTTGTGTGTGTTGT -NH_2 - 3'

SEQ ID No. 4:

5' -GTGTGTTGTGGGGGGTGGGGGTGTTGTGTGTGTTGT -Chol. - 3'

Control for SEQ ID No. 5:

5'-GTTGGTGTTGGTGGGGTGGTGGTGGTGGTGGTGGTTGTGGT-NH_2-3'

SEQ ID No. 5:

5' -GTTGGTGTTGGTGGGGTGGTGGTGGTGGTGGTTGTGGT -Chol. -3'

Attachment of cholesterol to the 5' end of oligonucleotides

Cholesteryl phosphoramidite was used on the synthesizer for this purpose. The presence of dimethoxytrityl group in the product of Example 7 reduces the coupling yield considerably. Another cholesteryl phosphoramidite that does not contain the dimethoxytrityl group was prepared as described in Example 7. The presence of cholesterol at the 5' end was confirmed by the elution behavior of this compound on HPLC column as well as its decreased mobility on gel as compared to the unmodified oligonucleotide.

EXAMPLE 12

Post synthetic modification of oligonucleotides with cholesterol linker containing reversible disulfide linkage

(Scheme 4)

The scheme for this synthesis was based on the procedure developed by Oberhauser and Wagner, Nucl. Acids Res. 20, 533-8 (1992). The oligonucleotide of SEQ ID No.1 was modified with cholesterol at the 3' end using this procedure.

1) Modification with dithiopyridine: A solution of oligonucleotide of SEQ ID No.1 (67 nmole), 38mer modified with (3-amino-2hydroxy-propyl)phosphate at the 3'-end, in 50 mM HEPES buffer (0.75 ml,

pH 7.9). To the mixture was added N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, 4.2 mg, 13.4 μmole, 200 equivalents, Pharmacia) in 300 μl ethanol. The mixture was stirred for 4 hours at room temperature. The reaction mixture was then subjected to gel filtration on Sephadex G25 (NAP-25, Pharmacia) with 20 mM HEPES buffer (pH 7.3) as eluent. The first fractions containing the oligonucleotides were evaporated to dryness in a Speedvac, and re-dissolved in 200 μl of water. The crude product was purified by HPLC (Nucleosil RP-18 column, 250x4 mm; buffer A: 100 mM triethyl ammonium acetate, pH 6.5; buffer B: acetonitrile; gradient: 0-40 minutes, 0-40% B) to provide the product.

2) PMDBD-salt formation: A solution of the product of 1) above (37 nmole) in 250 μl water was added to 250 μl of a solution of 4 mg PMDBD (3,3,6,9,9-pentamethyl-2,10-diazabicyclo(4.4.0)dec-1-ene,Fluka)carbonatesalt in 50% aqueous methanol. The solution was then diluted with water to a volume of 3 ml and freeze dried. The lyophylisate was re-dissolved in 1 ml of methanol:water (1: 4) and subjected to gel filtration on Sephadex G25 (NAP-25, Pharmacia), eluted with methanol:water (1:4). The fractions containing oligonucleotide were concentrated in a speedvac to remove methanol. The residue was re-dissolved in 1 ml of methanol : dichloromethane (1:2).

3) Conjugation to thiocholesterol: To 0.5 ml of the solution (16 nmole of the product from (2)) was added to methanol (200 μl), 180 mM methanolic PMDBD trifluoroacetate buffer pH 9 (30 μl) and thiocholesterol (600 μg, 1.5 μmole, Sigma) in 300 μl dichloromethane. The reaction mixture was kept under argon for 20 hours at room temperature. The solution was

evaporated to dryness in a speedvac, re-dissolved in 400 μl methanol chloroform (1:1), and extracted with water (100 μl x 8 times). The aqueous extracts containing the oligonucleotide were combined and concentrated to 500 μl in a speedvac to remove methanol. The material was purified by reverse phase High Performance Liquid Chromatography (HPLC).

Characterization of cholesteryl modified oligonucleotides

Cholesteryl modified oligonucleotides were characterized by HPLC, polyacrylamide gel electrophoresis (PAGE), CE (Capillary electrophoresis), base composition and NMR. The elution time for these compounds is about 10 minutes higher than unmodified oligonucleotides even on ion-exchange chromatography on Q-Sepharose columns. The mobility is slowed on PAGE compared to unmodified oligos. Proton NMR shows the presence of a cholesteryl fragment. The binding constant is essentially the same as that of unmodified oligonucleotide in triple helix formation.

EXAMPLE 13

Radiolabeling of Oligonucleotides

Prior to uptake experiments oligonucleotides were radiolabeled at the 5' end using polynucleotide kinase and g^{35}S-ATP (Amersham). Oligonucleotide was separated from unreacted ATP by chromatography on a NAP-5 column (Pharmacia).

EXAMPLE 14

Cell Culture

HeLa, Vero, and U937 cell lines were obtained from the American Type Culture Collection. HeLa and Vero cells were maintained in Modified Eagles Medium with 10% heat inactivated fetal bovine serum (FBS). U937 cells were maintained in RPMI containing 10% FBS. In both cases, the cells were grown at 37° C in an atmosphere of 95% air/5% CO2.

EXAMPLE 15

Measurement of Cellular Uptake

For cell uptake experiments, HeLa and Vero cells were grown to near confluence in 75 cm^2 Corning tissue culture flasks. Prior to the experiment cells were trypsinized and transferred to 24 well dishes at a density of 100,000 cells per well. After incubation for 24 hours at 37°C, $2x10^6$ cpm of labeled oligonucleotide was added to each well and unlabeled oligonucleotide was added to a final concentration of 1 μM. Cells were harvested at several points between 1 hour and 24 hours as follows. Wells were rinsed 4x with PBS (GIBCC)), suctioning between rinses. After the 4th wash was removed, 200 μl of 0.25% trypsin was added and removed to rinse monolayer. Then 200 μl trypsin was added again and removed. Plates were then incubated at 37° for 2 minutes in CO_2 incubator and cells were immediately resuspended in 600 μl of media containing 10% FBS. The resuspended cells were transferred to 1.5 ml tube, spun in a microcentrifuge at 1000xg at room temperature for 6 minutes to pellet. The supernatant was

removed and discarded and the pellet was resuspended in 500 μl of PBS and recentrifuged 3 times.

U937 cells (which grow in suspension) were added to 24 well dishes at a density of 1 x 106 cells/well and labeled and unlabeled oligonucleotide were added immediately as described above. To harvest, U937 cells were transferred to 1.5 ml tubes and washed 4 times by centrifugation with PBS as described above.

For "whole cell" determinations the pellet was resuspended in 500 μl PBS and removed to a scintillation counting vial. For "nuclear" fractions, the whole cell pellet was resuspended in 500 μl of lysing buffer at 4° and incubated on ice for 5 minutes and centrifuged as above to separate the pellet (nuclei) from the supernatant (cytoplasm). The nuclear pellet fraction was washed again with lysing buffer, centrifuged, and resuspended in 500 μl of PBS. All samples were then analyzed by liquid scintillation counting.

All cell counts were performed using a hemocytometer. Cell and nuclear volumes, used to estimate the intracellular oligonucleotide concentration, were determined using a Coulter Channelizer (Coulter Electronics).

EXAMPLE 16

Figures 5-8 illustrate the results of 4 experiments in which the cellular uptake of 2 different oligonucleotides were determined. These results demonstrate that 3'-amine modified oligonucleotides are accumulated by HeLa, Vero and U937 cells such that the internal concentration is equal to

or exceeds that of the culture media (1 μM). Cholesterol modified oligonucleotides, however, reach concentrations that exceed that of the media by 10-50 fold. Thus, attachment of cholesterol to the 3' end of oligonucleotides can enhance the degree of uptake of oligonucleotides. Table 1 shows the "enhancement factor" calculated by dividing the intracellular concentration of the 3'-cholesterol oligonucleotide by that of the 3'-amine derivative.

TABLE 1. Enhancement of cellular uptake by cholesterol modification.

Oligonucleotide	Cell Line	Whole Cell	Nucleus	Time Point
SEQ ID No. 1/control	Vero	6	10	24
SEQ ID No. 1/control	U937	2	3	24
SEQ ID No. 1/control	HeLa	22	26	4
SEQ ID No. 3/control	HeLa	5	5	4

EXAMPLE 17

Enhanced Activity of 3'-Cholesterol Oligonucleotides

The increase in intracellular and intranuclear concentration of the 3'-cholesterol oligonucleotides appears to translate into an enhancement in biological activity. The oligonucleotide of SEQ ID No.4 was designed to block expression of a synthetic gene promoter which controls the expression of a chloramphenicol acetyl transferase reporter gene. The unmodified oligonucleotide of SEQ ID No. 4 did not inhibit the CAT gene transfection assays at media concentrations up to 40 μM. In contrast, the 3'-modified

oligonucleotide, SEQ. ID No. 4, produced a 40% inhibition of gene expression at 20 μM.

SYNTHESIS OF CHOLESTERYL SUPPORTS AND PHOSPHORAMIDITE USING 6-AMINOHEXANOIC ACID SPACER

Figure 9 illustrates the synthesis of the compounds described in Examples 18-25.

EXAMPLE 18

Synthesis of 2-N-(9-fluorenylmethyloxycarbonyl) amino-1,3-propaneglycol.

2-Amino-1,3-propanediol, (50g, 54.9 mmole) was dissolved in anhydrous DMF (40 mL) and distilled diisopropylethylamine (11.45 mL, 65.85 mmole) and the solution was cooled in an ice-cold water bath. To the stirred solution was added 9-fluorenylmethylchloroformate (15.63 g, 60.35 mmole). The reaction mixture was continuously stirred fast for 15 minutes at 0°C and then 1 hour at room temperature. The resulting reaction mixture was poured into an ice-cold saturated aqueous sodium bicarbonate solution (600 mL). The white precipitate was filtered and washed thoroughly with water. The precipitate was recrystallized in ethyl acetate to provide the product, 2-N-(9-fluorenylmethyloxycarbonyl) amino-1,3-propaneglycol (15.98g, yield: 94%) as a white solid. $_1$HNMR ($CDCl_3$):∂

EXAMPLE 19

Synthesis of 1-O-(4,4'-Dimethoxytrityl)-2-N-(9-fluorenylmethyloxycarbonyl) aminopropaneglycol.

Design and Synthesis of Cholesteryl Oligonucleotide Containing Oligo-Cytidine Linker.

2-N-(9-fluorenylmethyloxycarbonyl) aminopropaneglycol (7.0g, 22.35 mmole) and 4-dimethylaminopryidine (0.54g, 4.4 mmole) were dissolved in anhydrous pyridine (20 mL) and distilled CH_2Cl_2 (200 mL). The solution was cooled to -20 to -15°C. To the stirred solution was added dropwise a solution of 4, 4'-dimethoxytritylchloride (7.57g, 22.35 mmole) in CH_2Cl_2 (50 mL). The solution was stirred at the above temperature for 1 hour and at 0°C for 30 minutes. Finally, methanol (10 mL) was added to the mixture and stirred for 15 minutes. The resulting reaction mixture was extracted with saturated $NaHCO_3$ aqueous solution (100 mL, twice) and brine (100 mL, twice). The organic phase was dried over anhydrous $MgSO_4$ and concentrated to give a light yellow oil which was purified by silica gel column chromatography using a gradient of CH_2Cl_2 and CH_2Cl_2:MeOH 19:1, as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide the product.

EXAMPLE 20

Synthesis of 1-O-(4,4'-Dimethoxytrityl)-2-aminopropaneglycol.

1-O-(4,4'-Dimethoxytrityl)-2-N-(9-fluorenylmethyloxy)carbonyl-2-amino-1,3-propanediol, was dissolved in CH_2Cl_2 (100 mL) and piperidine (50 mL). The solution was stirred at room temperature for 2 hours. Then, the resulting reaction mixture was concentrated to dryness. The crude material was purified by silica gel column chromatography using CH_2Cl_2 : Et_3N, 8:1:1, as the eluents. The homogeneous fractions were combined and concentrated under reduced pressure to a volume of 100 ml to provide the product, 1-O-(4,4'-Dimethoxytrityl)-2-aminopropaneglycol.

EXAMPLE 21

Synthesis of N-(cholesteryloxy)carbonyl-6-aminohexanoic acid.

6-Aminohexanoic acid (1.31g, 10 mmole) was suspended in anhydrous DMF (25 mL) and bis(trimethylsilyl)acetamide (8.9 mL, 31 mmole). The mixture was stirred until the acid dissolved completely, for about 3 hours. Then, the mixture was cooled to -20 to -15°C, and to the mixture was added N-methylmorpholine (1.1 mL, 10 mmole) and cholesteryl chloroformate (4.49 g, 10 mmole). The mixture was stirred for 1 hour and then, at 0°C for 30 minutes. The resulting reaction mixture was concentrated to dryness under reduced pressure. The residue was re-dissolved in CH_2Cl_2 (350 mL) which was washed with 4% HCl (100 mL, twice), dried on Na_2SO_4 and concentrated to a heavy oil. The crude material was purified by silica gel chromatography using CH_2Cl_2 : MeOH 19 : 1. The homogeneous fractions were combined and concentrated under reduced pressure to provide the

product, N-(cholesteryloxy)carbonyl-6-aminohexanoic acid (5.22 g, yield: 92%) as a white solid.

EXAMPLE 22

Synthesis of 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-aminohexanoyl)aminopropaneglycol.

N-(cholesteryloxy) carbonyl-6-aminohexanoic acid (2.4g, 4.4 mmole) was dissolved in anhydrous DMF (30 mL). To the solution was added N-hydroxysuccinimide (0.61g, 5.3 mmole) and 1,3-dicyclohexylcarbodiimide (1.09 g, 5.3 mmole). After stirring at room temperature overnight, the reaction mixture was filtered to remove the white precipitate, and the filtrate was added to a solution of 1-O-(4,4'-Dimethoxytrityl)-2-aminopropaneglycol (17, c.a. 4.4 mmole) in CH_2Cl_2 : MeOH : Et_3N, 8:1:1 (80 mL0. After stirring at room temperature overnight, the mixture was extracted with saturated $NAHCO_3$ aqueous solution (100 mL, twice), brine (100 mL, twice), the organic phase was dried over anhydrous $MGSO_4$ and concentrated to dryness to remove the solvent to give a light yellow oil which was purified by silica gel column chromatography using a gradient of CH_2Cl_2 and CH_2Cl_2 : MeOH, 19:1, as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide the product, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-aminohexanoyl)aminopropaneglycol,2.8g,yield : 69.2%).

EXAMPLE 23

Synthesis of 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-aminohexanoyl)-3-O-succinate aminopropaneglycol.

1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-amino hexanoyl)aminopropaneglycol was dissolved in anhydrous pyridine. To the solution was added succinic anhydride (0.51 g, 5.13 mmole) 4-dimethylaminopyridine (0.33 g, 27. mmole). The mixture was stirred for 2 hours. The reaction mixture was concentrated to an oil to remove pyridine. The oil was re-dissolved in dichloromethane (200 ml), washed with 10% aqueous citric acid solution (150 ml, twice) and water (150 ml, twice). The organic phase was dried over anhydrous NA_2SO_4. After concentration, the crude oil was purified by silica gel column chromatography using a gradient of CH_2Cl_2 and CH_2Cl_2 : MeOH, 19:1, as the eluent. The homogeneous fractions were combined and concentrated under reduced pressure to provide the product (2.34 g, yield: 70%) as a white solid foam.

EXAMPLE 24

Synthesis of cholesteryl CPG and cholesteryl Tenta Gel supports.

1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-amino hexanoyl)-3-O-succinate aminopropaneglycol, O-benzotriazole-1-yl-N,N,N',N'-tetramethyluoronium-tetrafluoroborate (1.05g, 3.26 mmole), 1-hydroxy benzotriazole hydrate (0.44 g, 3.26 mmole), N-ethyl morpholine (0.37g, 3.26 mmole) were dissolved in anhydrous DMF (76ml). After 5

minutes of shaking, to the mixture was added tentagel-NH_2 (10g, loading capacity:220 umole/g). The reaction mixture was swirled slowly for 3 hours. Capping and washing steps were done as described above. The loading of cholesterol on tentagel support was estimated to be 152umole/gram.

Cholesteryl CPG support was prepared using the above procedure with aminopropyl derivertized CPG support. The loading was estimated in μmole/gram.

EXAMPLE 25

Synthesis of 2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-amino hexanoyl)aminopropaneglycyl phosphoramidite.

1-O-(4,4'-dimethoxytrityl)-2-N-(N-cholesteryloxy)carbonyl-6-amino hexanoyl)aminopropaneglycol (2.8g, 3.04 mmole) was dissolved in anhydrous dichloromethane (30 mL). To the solution was added diisopropylethylamine (1.0 mL, 6.1 mmole), and then, 2-cyanoethyl-N,N'-diisopropyl chlorophosphoramidite (0.82 mL, 3.64 mmole). After stirring for 1 hour at room temperature, the solution was extracted with 5% equeous $NAHCO_3$ solution (50 mL, twice) and brine (50 mL, twice). The solvent extract was dried on Na_2SO_4 and concentrated to a heavy oil which was purified by silica gel column chromatography using n-hexane : ethyl acetate : triethylamine, 20 : 20 : 1 as an eluant.

SYNTHESIS OF CHOLESTERYL SUPPORTS AND PHOSPHORAMIDITE USING GLYCYLGLYCYLGLYCINE SPACER

Figure 10 shows the synthetic scheme for the compounds described in Examples 26-30.

EXAMPLE 26

Synthesis of N-(cholesteryloxycarbonyl)glycylglycylglycine.

Glycylglycylglycine (5.5g, 29.1 mmole) was suspended in anhydrous DMF (90 mL), dichloromethane (90 mL) and bis (trimethylsilyl) acetamide (25.7 mL, 204.76 mmole). After stirring at room temperature, overnight, the mixture was cooled to -20 to -15°C. To the mixture were added N-methyl morpholine (3.8 mL, 34.9 mmole) and a solution of cholesteryl chloroformate (15.9g, 34.9 mmole) in CH_2Cl_2 (90 mL). The mixture was stirred at cold temperature for 2 hours and at 0°C for 30 minutes. The mixture was concentrated under reduced pressure to dryness yield: 80.5%.

EXAMPLE 27

Synthesis of 1-O-(4,4'-dimethoxytrityl)-2-N-((N-cholesteryloxycarbonyl) glycylglycylglycyl)aminopropaneglycol.

1-O-(4,4'-Dimethoxytrityl)-2-N-((N-cholesteryloxycarbonyl) glycylglycylglycyl)aminopropaneglycol was synthesized using the procedure employed for the synthesis of compound 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-aminohexanoyl)aminopropaneglycol.

EXAMPLE 28

Synthesis of 1-O-(4,4'-dimethoxytrityl)-2-N-((N-cholesteryloxycarbonyl) glycylglycylglycyl)-3-O-succinate aminopropaneglycol.

1-O-(4,4'-Dimethoxytrityl)-2-N-((N-cholesteryloxycarbonyl) glycylglycylglycyl)-3-O-succinate aminopropaneglycol was synthesized using the succinylation procedure of compound 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-aminohexanoyl)-3-O-succinate aminopropaneglycol.

EXAMPLE 29

Synthesis of cholesteryl CPG and cholesteryl Tenta Gel supports.

Cholesteryl CPG and cholesteryl TentaGel supports were synthesized using the preparation procedure for compounds cholesteryl CPG and cholesteryl Tenta Gel.

EXAMPLE 30

Synthesisof2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2-N-((N-cholesteryloxycarbonyl) glycylglycylglycyl)aminopropaneglycyl phosphoramidite.

2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2N-((N-cholesteryloxycarbonyl) glycylglycylglycyl)aminopropaneglycyl phosphoramidite (31) was synthesized using phosphorylation procedure of compound 2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-amino hexanoyl)aminopropaneglycyl phosphoramidite.

Figure 11 shows the synthesis of compounds that have a longer spacer between the phosphate and cholesterol as well as the hydroxyl and the cholesterol.

EXAMPLE 31

Synthesis of cholesteryl TFOs using the CPG support, 2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxy)carbonyl-6-amino hexanoyl)aminopropaneglycyl phosphoramidite.

Several cholesteryl TFOs were synthesized on an ABI 380B or 394 automated synthesizer under conditions described in Example 11 above. The coupling yields were ≥ 98% at each step including the first step. The cholesteryl TFOs were purified as described in Example 11. The TFO with the cholesterol at the 3' end containing the new linkers elutes about 10 minutes earlier than the TFO with cholesterol containing 3-aminopropyl solketal linker. The cholesteryl TFOs also dissolve ~ 10 times more readily in water.

The following sequences were synthesized with this new cholesterol version at the 3' end.

5' GTGGTGGTGGTGTTGGTGGTGGTTTGGGGGGTGGGG3'-cholesterol (B-106-85, anti-HSV 2 TFO) (SEQ ID No. 11)

5' GTGGTTGGTGGTGGTGTGTGGGTTTGGGGTGGGGGG3'-cholesterol (B-106-86, control for B-106-85) (SEQ ID No. 12)

5' GGTGGTTGGGGGGTGGGGGGG3'-cholesterol (B-133-54. anti-HSV2 TFO) (SEQ ID No. 13)

5' GGGTGGGGTGGTGGGTGGGG3'-cholesterol

(B-133-55, control for B-133-54) (SEQ ID No. 14)

HPLC profile of B-133-55 is shown as an example in Figure 12.

The following TFOs were synthesized using the cholesteryl phosphoramidite, synthesis of 2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2-N-((N-cholesteryloxycarbonyl) glycylglycylglycyl)aminopropaneglycyl phosphoramidite.

Cholesterol 5'
TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGGTG3'-propanol amine (A-100-20, anti-HIV TFO) (SEQ ID No. 15)

Cholesterol 5'
GTGGTGGTGGTGTTGGTGGTGGTTTGGGGGGTGGGG3'-propanolamine (B-106-89, anti-HSV 2 TFO) (SEQ ID No. 16)

Cholesterol 5'GTGGTTGGTGGTGGTGTGTGGGTTTGGGGTGGGGGG3'-(B-106-90, control for B-106-89) (SEQ ID No. 17)

The coupling yield at each step including the coupling of cholesterol for these TFOs was, 98.89%, 98.80% and 98.60%, respectively, indicating high-efficiency coupling with cholesteryl phosphoramidite. The TFOs were also labeled easily and the analysis of labeled TFOs on gel is shown in Figure 13. B-106-78 in lane 1 contains cholesterol with 3'-aminopropyl solketal linker. The TFO does not enter the gel efficiently and

a lot of material is stuck in the well whereas the TFOs, B-106-90, B-106-89, B-106-85 and B-106-86 containing cholesterol with tri-glycyl and 6-aminohexanoic acid linkers in lanes 2, 3, 4 and 5 enter the gel efficiently. The latter TFOs did not show any difference in mobility compared to the 3' amine TFO as the TFO with 3-aminopropyl solketal linker.

EXAMPLE 32

Virus yield reduction assay

Vero cells (4 x 10^4/well) were seeded into 96-well culture plates in 0.1 mL of minimal essential medium with Earle salts (MEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a 5% CO_2 atmosphere. When the assay was begun, the media was removed and replaced with MEM/2%FBS containing oligonucleotide and returned to 37°C for the desired preincubation period (generally overnight). At the time of viral infection, the media/oligo was removed, the cells were rinsed 3X with MEM/2% and 50 mL of virus (10^5 pfu/mL for HSV-2) was added to the cells. The virus was allowed to adsorb for 10 minutes at 37°C. The virus was removed, the cells were rinsed 3X with MEM/2% (or once with PBS pH3, and twice with MEM/2%) and fresh MEM/2% with oligonucleotide was added to the wells. The cells were incubated for 14 hours at 37°C. When the media was removed, the cells were rinsed twice with 50 μl of MEM/2%, and 100 μl of MEM/2% was added to the wells. The plates were then placed at -70°C. Following a single freeze/thaw cycle the plates were titered in a standard fashion in 96 well plates.

TABLE 1

Viral Yield Assay Using 40 μM of Anti HSV-2 TFO's

	pfu/mL*	% Reduction in Titer
Virus	291,600	0
B-106-62 -3' amine	31,200	89
B-106-85 -3' cholesterol	19,200	93
B-106-71 -3' amine	80,100	73
B-106-86 -3' cholesterol	34,800	88

* Average of 3 assays.

The cholesterol modified anti HSV-2 TFO (B-106-85) has a slightly enhanced activity but the true TFO effect is masked by the oligonucleotide effect on adsorption of the virus as seen by the effect of the control TFO, B-106-71.

SYNTHESIS OF PHOSPHORAMIDITE CONTAINING LONGER PHOSPLATE LINKER

Synthesis of 6-N[1-O-(4,4'-dimethoxytrityl)-2-N(6-N(cholesteryloxycarbonyl)hexanoyl)amino-3-carbonyl]aminohexyl-1-ol (38)

Synthesis of linker CPG (39) and linker TentaGel (40) supports.

Synthesis of 2-cyanoethyl-N,N-diisopropyl-6-N[1-O-(4,4'-dimethoxytrityl)-2-N(6-N(cholesteryloxycarbonyl)hexanoyl)amino-3-carbonyl]aminohexyl phosphoramidite (41).

EXAMPLE 32

Improvements to enhance the nuclear uptake using reversible linkages

Uptake studies using fluorescein labeled cholesteryl modified TFOs (containing 3-aminopropyl solketal linker) indicate that a large proportion of the molecules are trapped in the endosomes or membranes. Release of these molecules allows further enhancement of entry into the nucleus and thus significant increase in nuclear uptake. This can be accomplished, for example, by reversible linkages between the TFO and the cholesterol moiety such that the TFO is cleaved after entry into the cell. Design and preparation of TFOs containing such linkages are described in Figures 14, 15, 16 and 17.

EXAMPLE 33

SYNTHESIS OF CHOLESTERYL SUPPORTS AND PHOSPHORAMIDITE WITH A LINKER CONTAINING DISULFIDE BONDS

Figure 14 shows the synthesis of N-(tert-butoxycarbonyl)-1,3-diaminopropane (42), the synthesis of 1-N-(cholesteryloxycarbonyl)-3-N-(tert-butoxycarbonyl) diaminopropane (43), the synthesis of 3'-((N-(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl dithio-3-propanoic benzyl ester (45), the synthesis of 3'-((N-(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl dithio-3-propanoic acid (46), the synthesis of 1-O-(4,4'-dimethoxytrityl)-2-N-(3'-(N-(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanoyl)aminopropaneglycol (47), the

synthesis of cholesteryl CPG (48) and cholesteryl TentaGel (49) supports, and the synthesis of 2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2-N-(3'-(N-(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanoyl)aminopropaneglycyl phosphoramidite (50).

Figure 15 shows the synthesis of 2'-Aminoethyl dithio-3-propanoic acid (51), the synthesis of 2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoic acid (52), the synthesis of 1-O-(4,4'-dimethoxytrityl)-2-N-(2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycol (53), the synthesis of cholesteryl CPG (54) and cholesteryl TentaGel (55) supports, and the synthesis of 2-cyanoethyl-N,N-diisopropyl-1-O-(4,4'-dimethoxytrityl)-2-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycyl phosphoramidite (56).

Compounds 48, 49, 50, 54, 55 and 56 (Figures 14 and 15) contain disulfide bonds in the linker. 50 and 56 are intermediates for attaching these linker-cholesterol to the 5' end and 48, 49, 54 and 55 facilitate the attachment at the 3' end. The disulfide linkage would be reduced inside the cell and release the TFO from cholesterol thus enhancing the entry into the nucleus.

TFOs containing multiple linker phosphates and cholesterol were prepared using compound 58 (Figure 16). Starting with any one of the following cholesterol-support (22, 23, 29, 30, 39, 40, 48, 49, 54, and 55) and by adding compound 58, successively a few times to the support followed by the sequence of the TFO, TFOs containing multiple phosphates were prepared. Compounds 59 a-e in Figure 16 are examples of such TFOs. Multiple phosphate linkages enhance the solubility of the TFO and are more

likely to be subjected to cleavage by phosphodiesterase inside the cell thus releasing the cholesterol and TFOs free.

Compounds 60 a-d in Figure 17, have an oligocytidine sequence (preferably 4-8 Cs) inserted between the TFO and the linker containing the cholesterol. A small stretch of Cs were susceptible to endonuclease. By inserting such a sequence, the TFO would be cleaved at Cs more readily inside the cell and the TFO can be released from the cholesterol. Deoxy or ribo Cs can be used.

It should be pointed out that a variety of combinations between the linkers, cholesterol and TFOs can be used for any sequence and one or more of such combinations might prove to be more effective than others.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

In conclusion, it is seen that the present invention and the embodiments disclosed herein are well adapted to carry out the objectives and obtain the end set forth in this application. Certain changes can be made in the method, sequences, compounds and chemicals without departing from the scope of this invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

What is claimed is:

1. A method of enhancing sequence specific binding of a synthetic triplex forming oligonucleotide (TFO) comprising the step of contacting said TFO with a cell, wherein said TFO comprises a nucleotide sequence of about at least 20 nucleotides long includes a G & T, is chemically modified with a lipophilic compound and is capable of binding to a DNA duplex target to form a triple helix.

2. The method of claim 1, wherein said lipophilic compound is selected from the group consisting of cholesterol, Vitamin E and 1,2-di-O-hexadecyl-3-glyceryl.

3. The method of claim 1, wherein said synthetic TFO has the sequence shown in TFO-Linker-cholesterol.

4. The method of claim 1, wherein said synthetic TFO has the sequence shown in TFO-X-Linker-cholesterol.

5. The method of claim 4, wherein said X is selected from the group consisting of multi-phosphate group and an oligo-cytidine group.

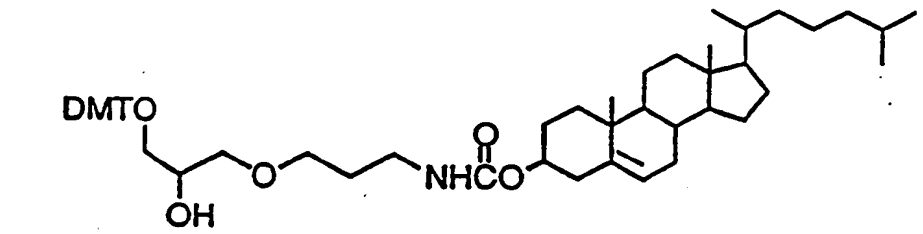
6. The method of claim 5, wherein said TFO containing a multi-phosphate group is selected from the group consisting of 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl(glycerol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)-6-

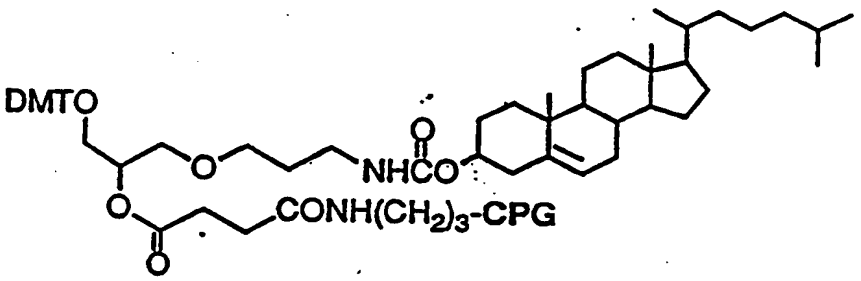
aminohexanoyl)aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)glycylglycylglycyl)-3-aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N(3'-N(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanoyl)aminopropaneglycol, and 1-O-(4,4',dimethoxytrityl)-2-N(2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycol.

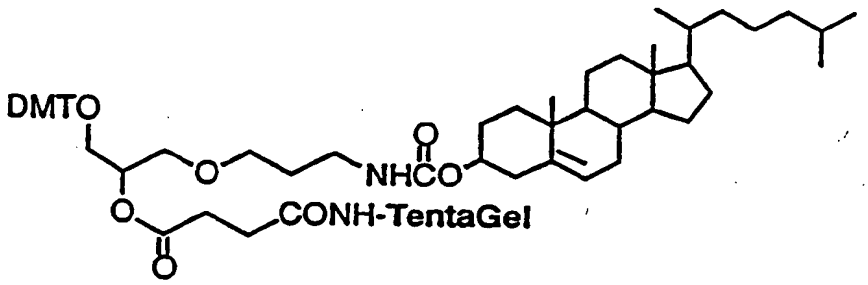
7. The method of claim 5, wherein said TFO containing an oligo-cytidine group is selected from the group consisting of 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glycerol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)-6-aminohexanoyl)aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)glycylglycylglycyl)-3-aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N(3'-(N-(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanyol)aminopropaneglycol, and 1-O-(4,4'-dimethoxytrityl)-2-N(2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycol.

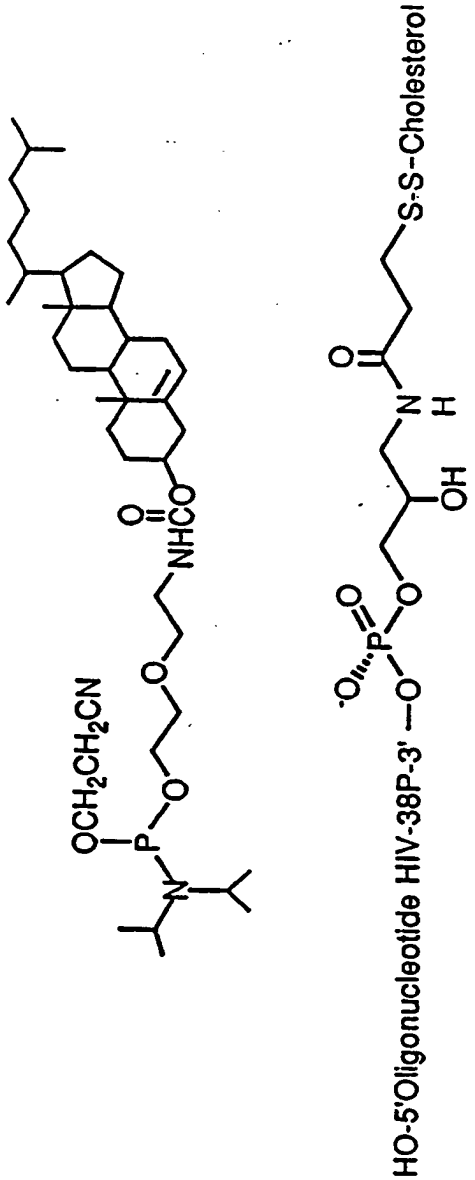
8. The method of claim 3, wherein said TFO is selected of SEQ. I.D. NOs. 1-5.

9. The method of claim 3, wherein said linker-cholesterol complex is selected from the group consisting of









10. The method of claim 1, wherein said target sequence is selected from the group consisting of a virus and an oncogene.

11. The method of claim 10, wherein said virus is selected from the group consisting of herpes simplex virus type 2 and human immunodeficiency virus.

12. The method of claim 10, wherein said oncongene is selected from the group consisting of c-myc and c-erb B2/neu(HER2).

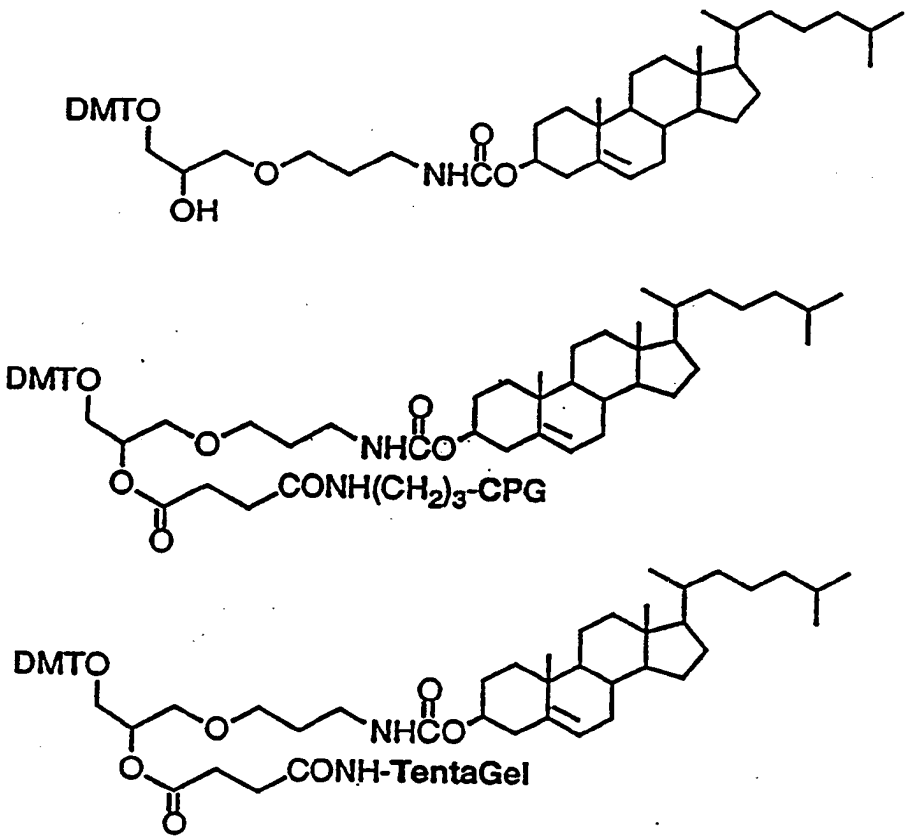
13. A method of treating a pathophysiological disease comprising administration of a triplex forming synthetic oligonucleotide (TFO) to an individual in an amount sufficient for cellular uptake and binding to a target sequence, wherein said TFO comprises a nucleotide sequence of about at least 20 nucleotides; said nucleotide sequence including G and T, is capable of binding to a DNA duplex target to form a triple helix, and has a lipophilic compound chemically attached to said TFO.

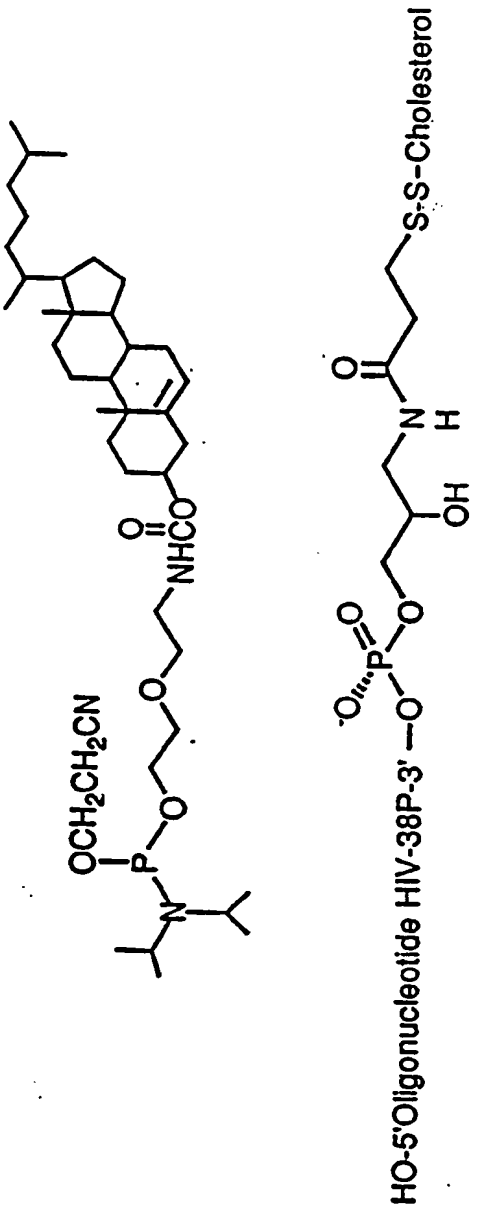
14. The method of claim 13, wherein said wherein said lipophilic compound is selected from the group consisting of cholesterol, Vitamin E and 1,2-di-O-hexadecyl-3-glyceryl.

15. The method of claim 13, wherein said synthetic TFO has the sequence shown in TFO-Linker-cholesterol.

16. The method of claim 13, wherein said TFO is selected from the oligonucleotides shown in SEQ. I.D. NO. 1-5.

17. The method of claim 15, wherein said linker-cholesterol is selected from the group consisting of





18. The method of claim 13, wherein said synthetic TFO has the sequence shown in TFO-X-Linker-cholesterol.

19. The method of claim 18, wherein said X is selected from the group consisting of multi-phosphate group and an oligo-cytidine group.

20. The method of claim 19, wherein said TFO containing a multi-phosphate group is selected from the group consisting of 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl(glycerol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)-6-aminohexanoyl)aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)glycylglycylglycyl)-3-aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N(3'-N(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanoyl)aminopropaneglycol, and 1-O-(4,4',dimethoxytrityl)-2-N(2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycol.

21. The method of claim 19, wherein said TFO containing an oligo-cytidine group is selected from the group consisting of 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glycerol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)-6-aminohexanoyl)aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl)glycylglycylglycyl)-3-aminopropaneglycol, 1-O-(4,4'-dimethoxytrityl)-2-N(3'-(N-(cholesteryloxycarbonyl)-1,3-diaminopropyl)propanoyl)dithio-3-propanyol)aminopropaneglycol, and 1-O-

(4,4'-dimethoxytrityl)-2-N(2'-N-(cholesteryloxycarbonyl)aminoethyl dithio-3-propanoyl)aminopropaneglycol.

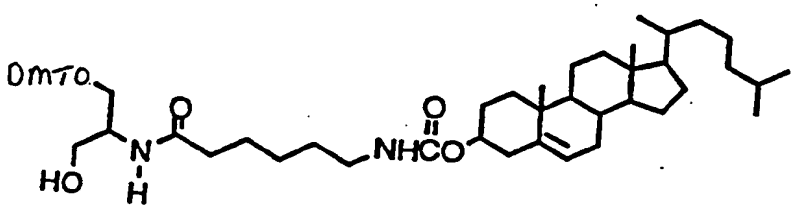
22. The method of claim 13, wherein said pathophysiological disease is selected from the group consisting of cell proliferative states and infection by a virus.

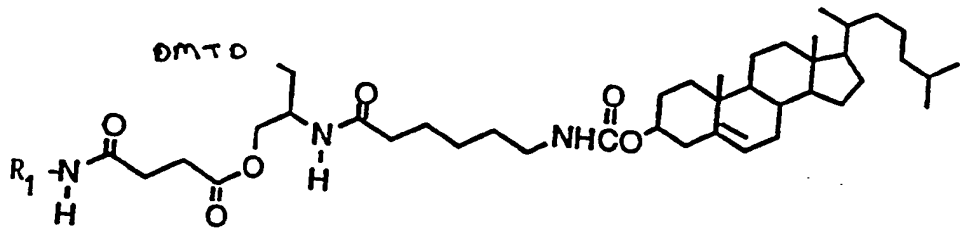
23. The method of claim 22, wherein said cell proliferative state is selected from the group consisting of breast cancer, lung cancer and cervical cancer.

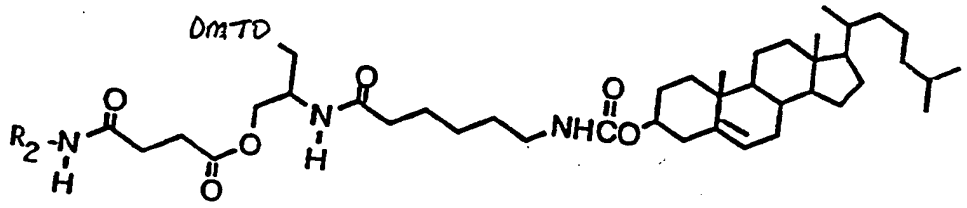
24. The method of claim 22, wherein said virus is selected from the group consisting of Herpes simplex virus type 2 and Human Immunodeficiency Virus.

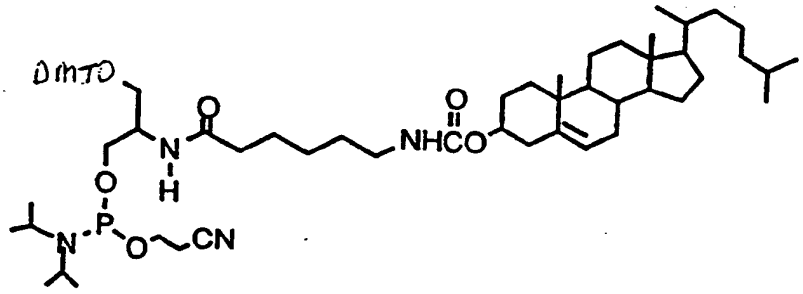
25. The method of claim 3, wherein said TFO is selected from SEQ ID Nos. 11, 13, 15 and 16.

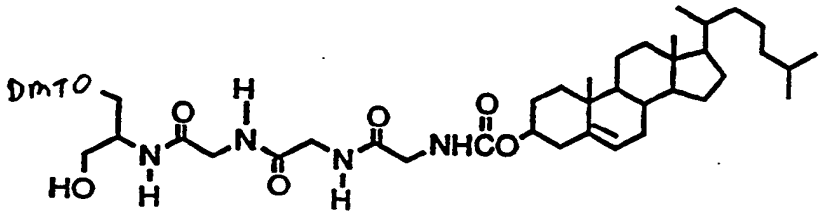
26. The method of claim 3, wherein said linker-cholesterol complex is selected from the group consisting of

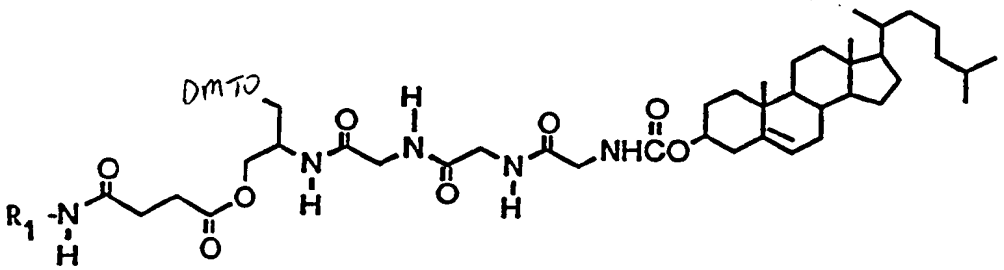


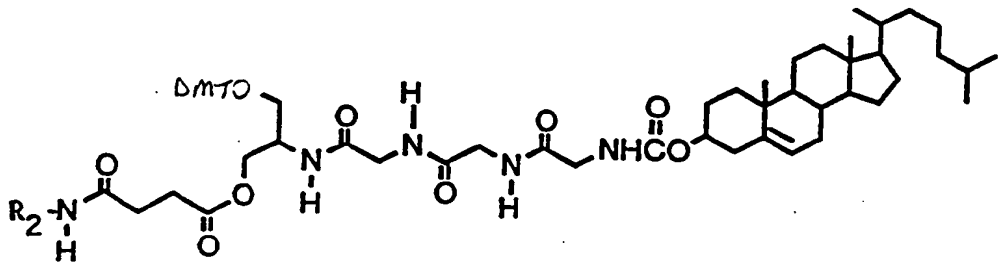


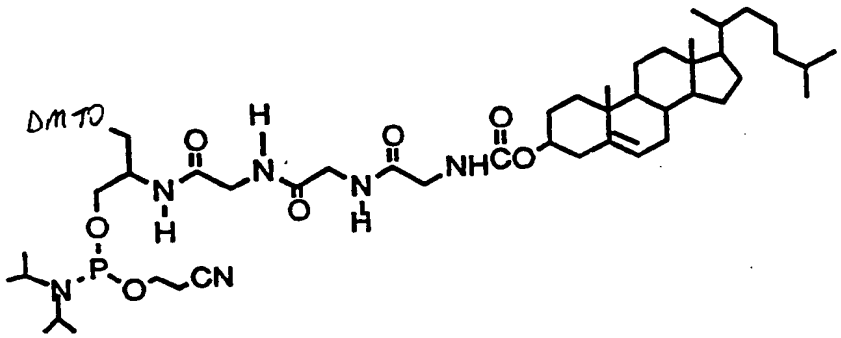


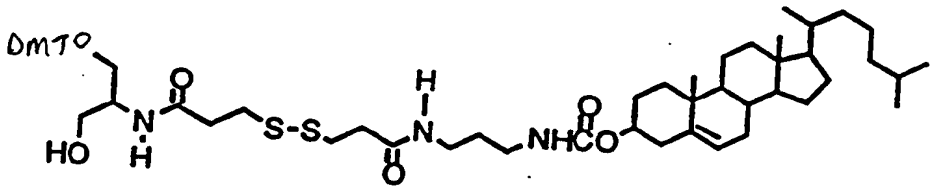


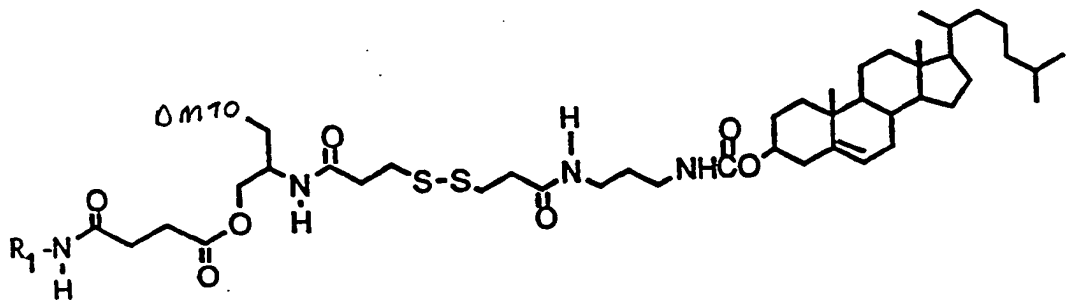


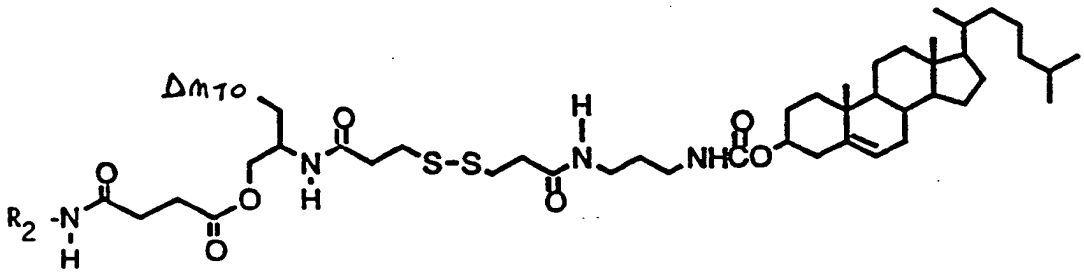


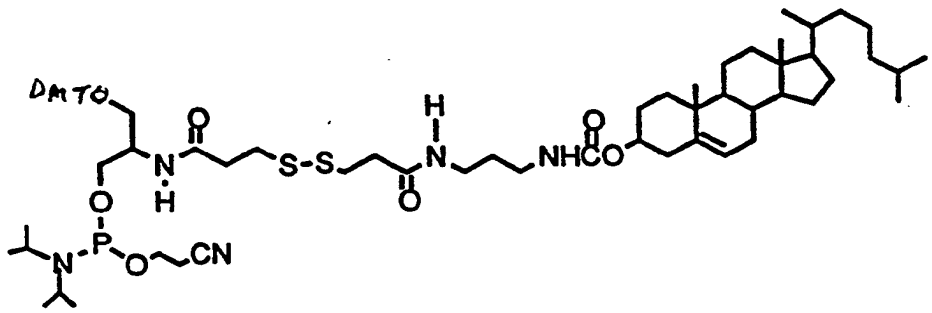


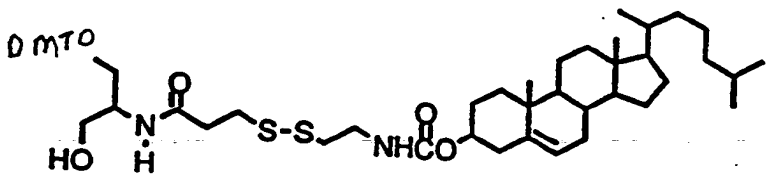


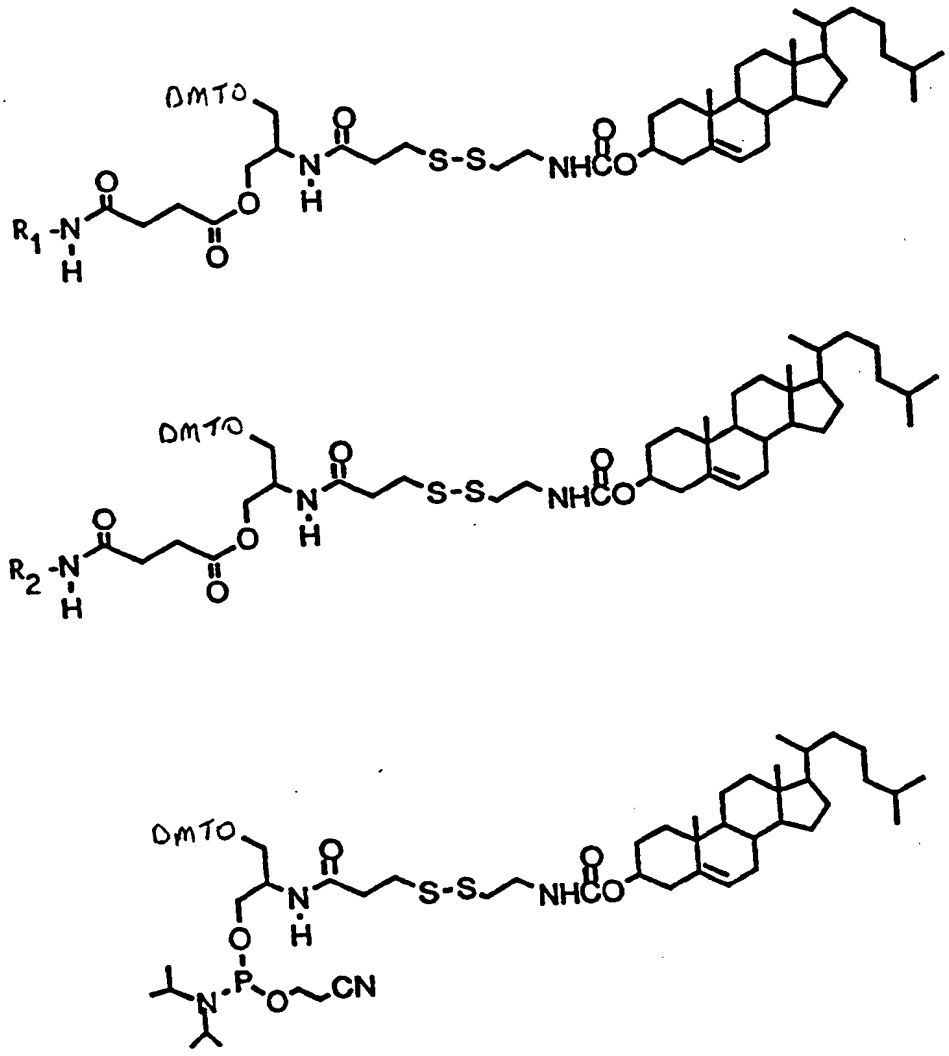








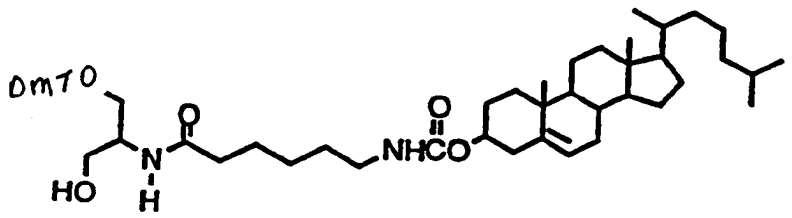


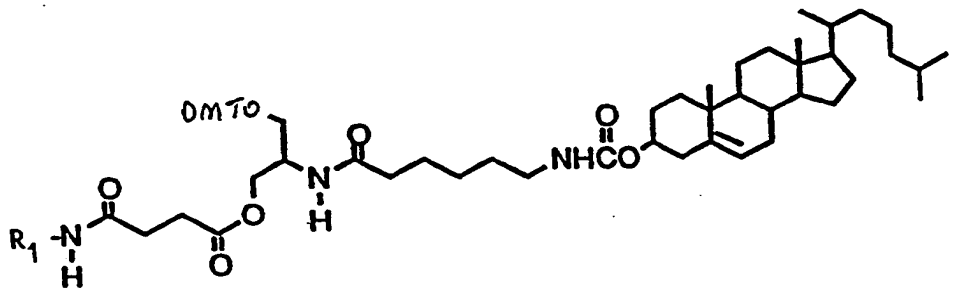


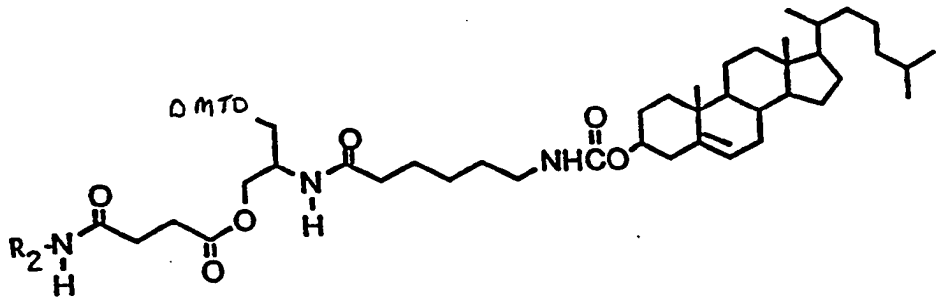
wherein R_1 is controlled pore glass and R_2 is TentaGel.

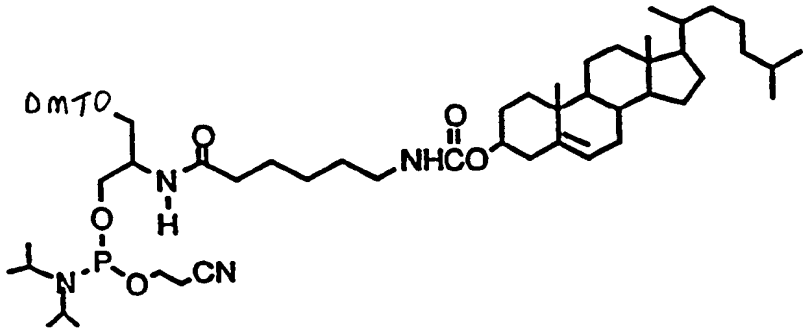
27. The method of claim 15, wherein said TFO is selected from SEQ ID Nos. 11, 13, 15 and 16.

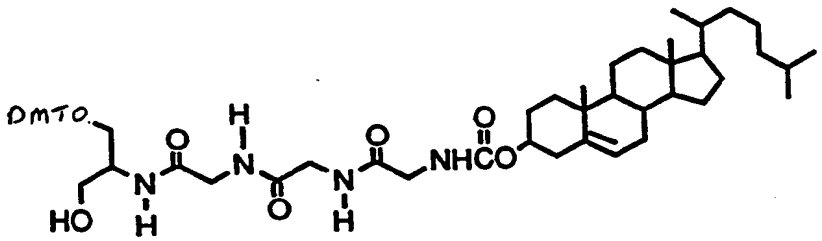
28. The method of claim 15, wherein said linker-cholesterol complex is selected from the group consisting of

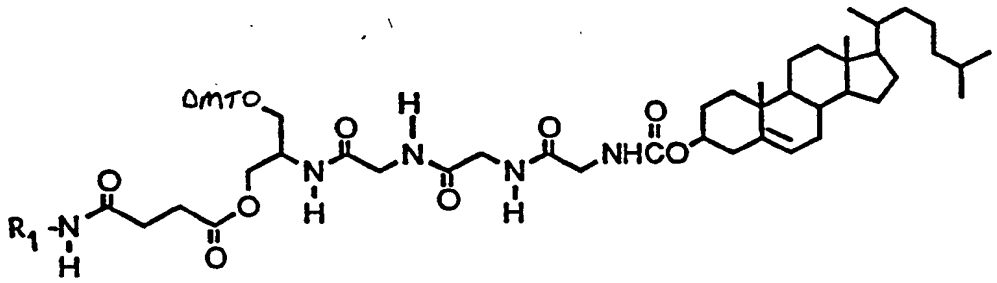


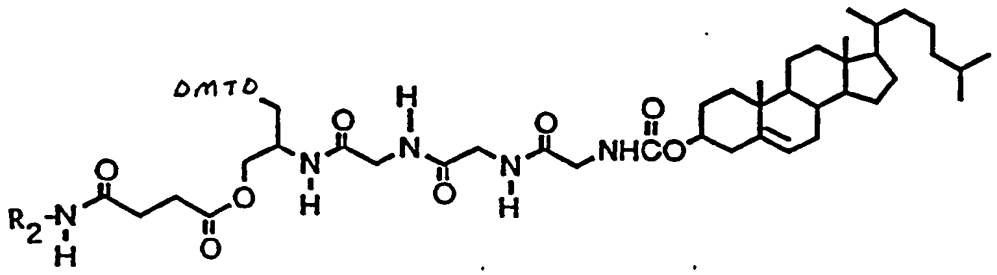


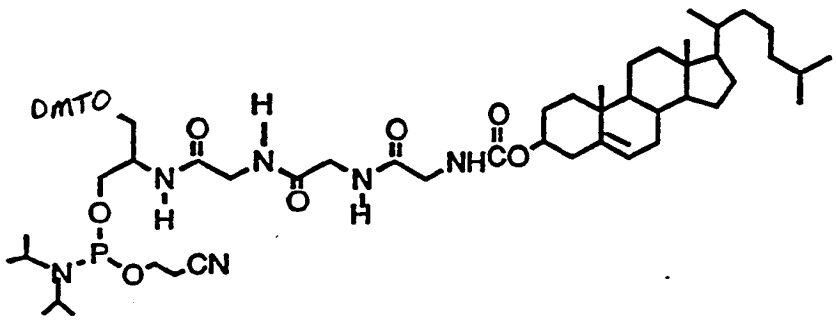


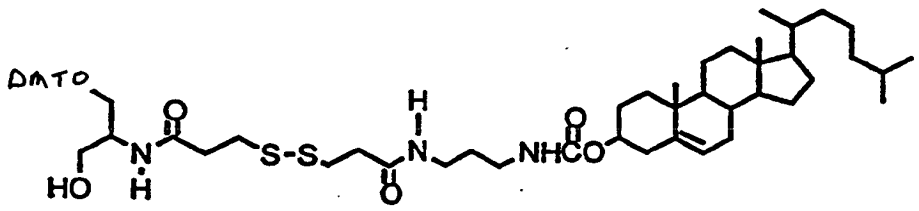


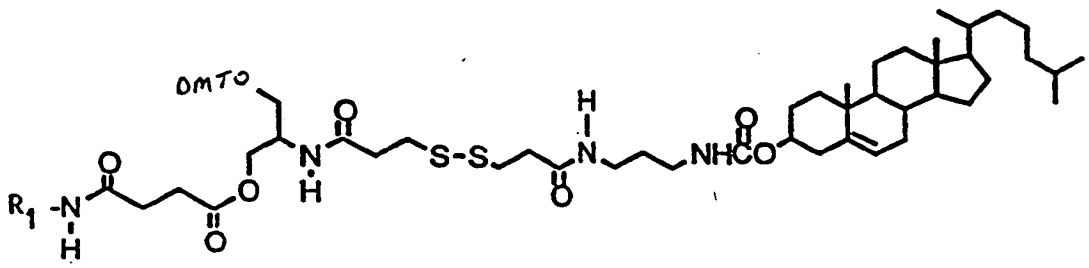


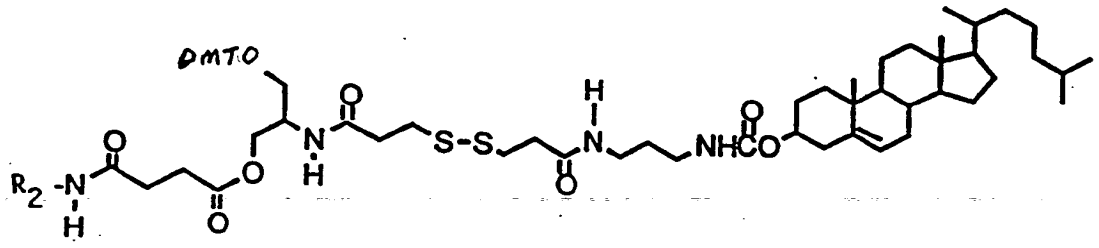


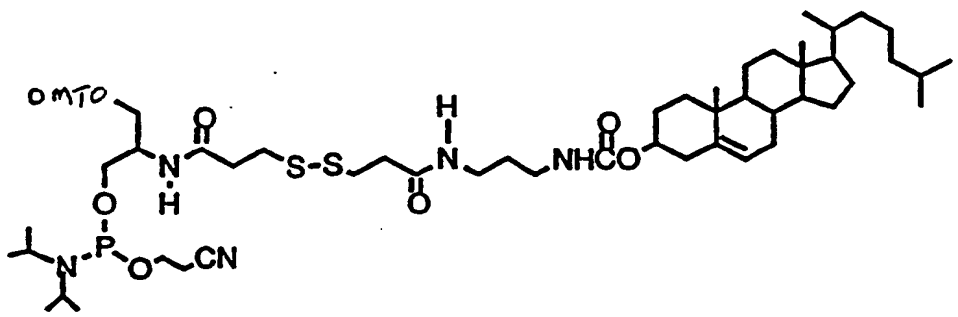


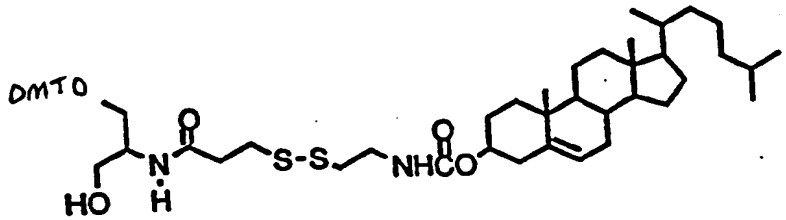


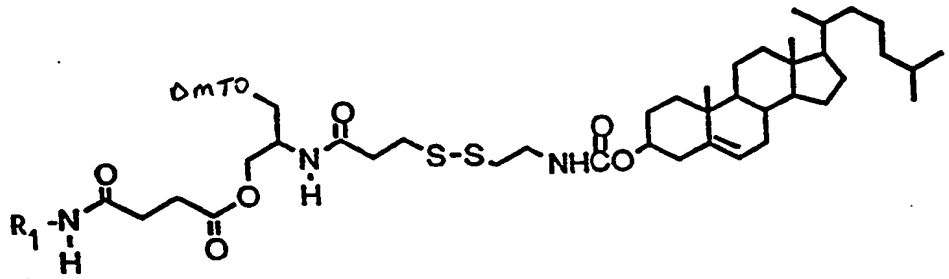


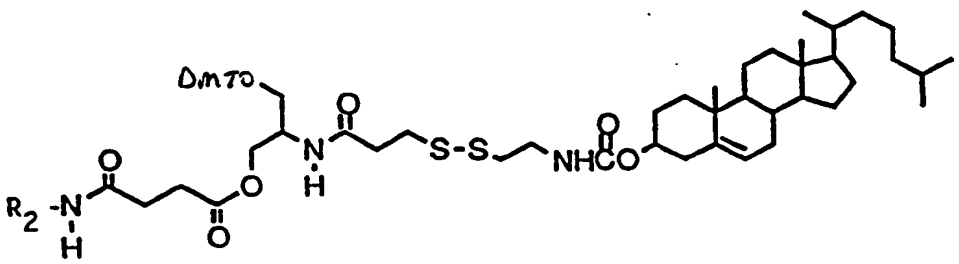


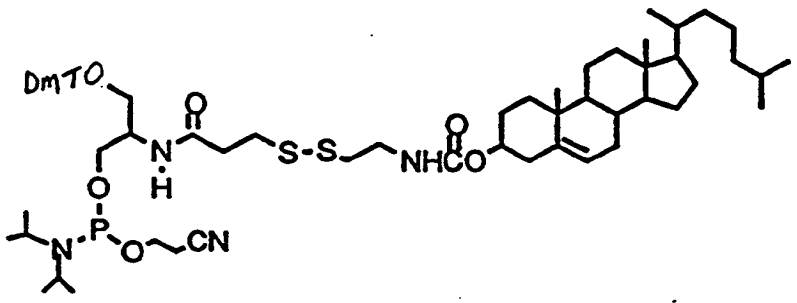












wherein R_1 is controlled pore glass and R_2 is TentaGel.

Scheme 1: Synthesis of cholesteryl linker

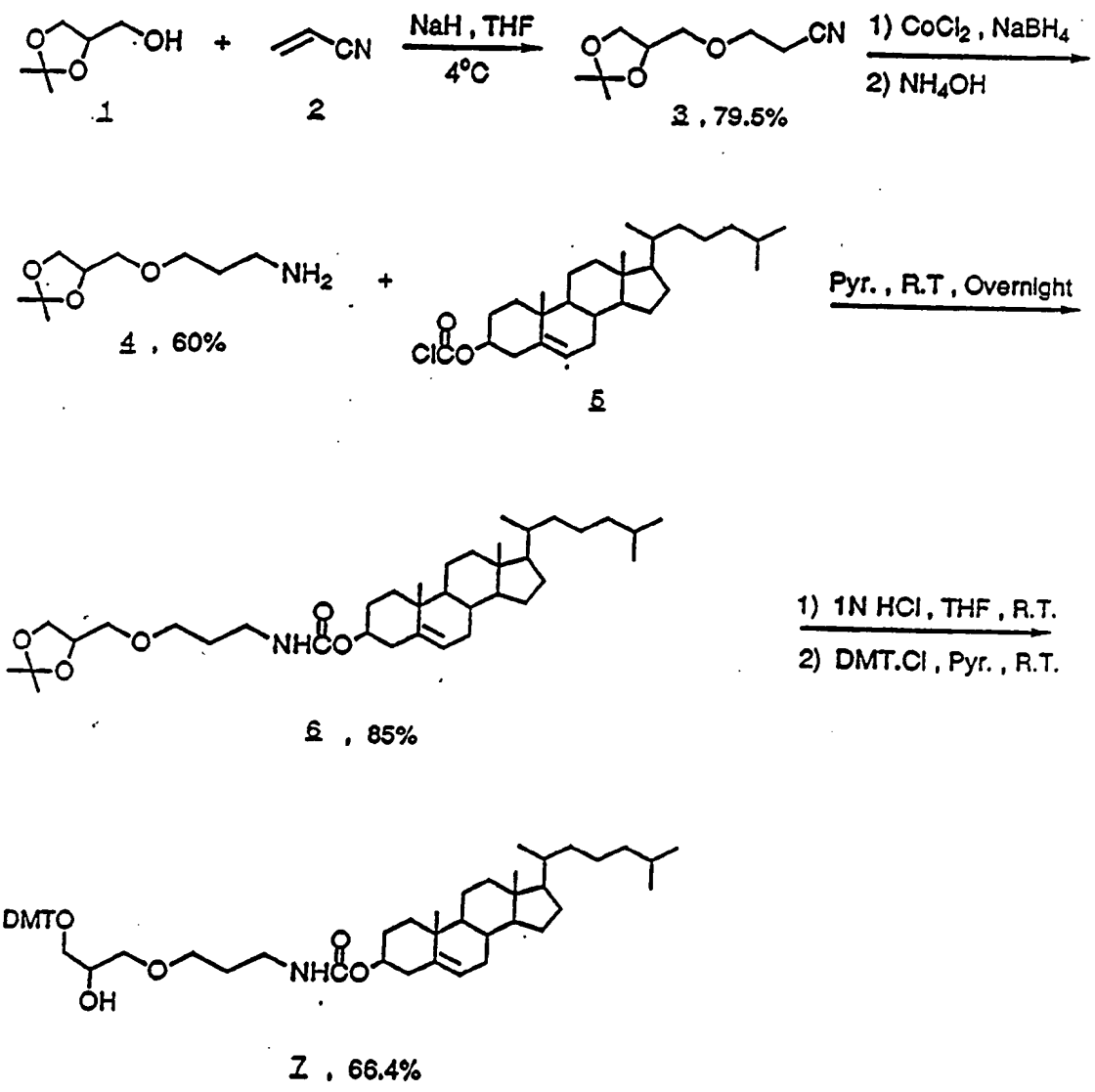
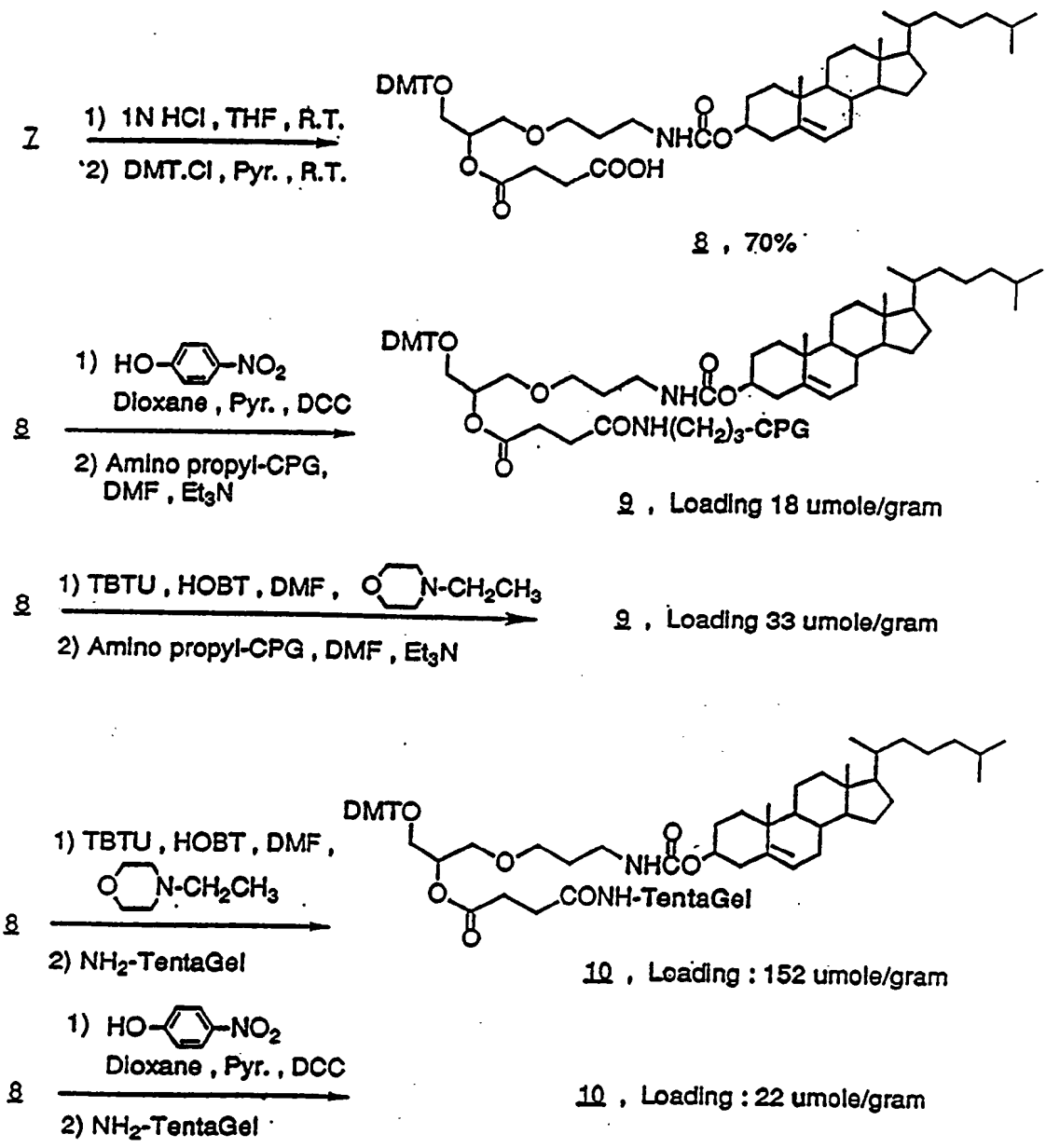


FIGURE 1

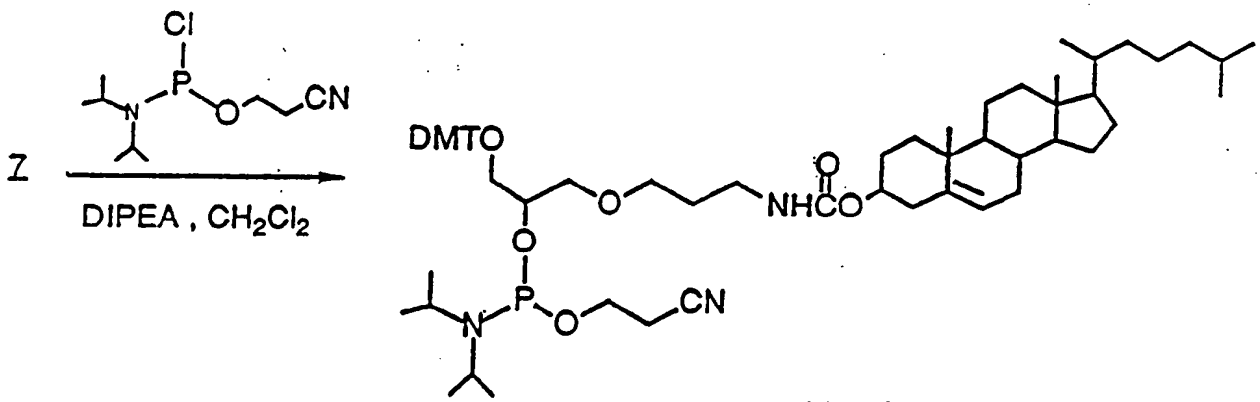
Scheme 2: Synthesis of cholesteryl-CPG and cholesteryl-TentaGel



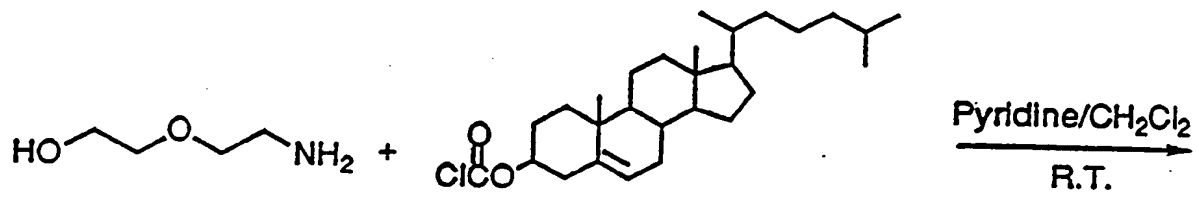
DCC = 1,3-Dicyclohexylcarbodiimide
TBTU = O-(1H-Benzotriazol-1-yl)-N,N,N',N'-tetramethyl uronium tetrafluoroborate
HOBT = 1-Hydroxy benzotriazole

FIGURE 2

Scheme 3: Synthesis of cholesteryl phosphoramidites



11 , 70%



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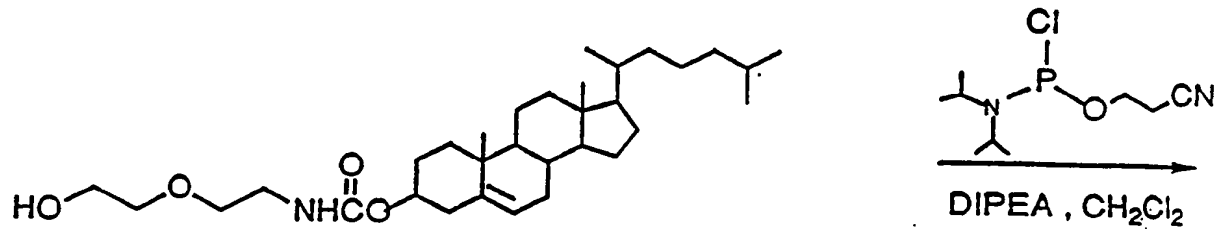

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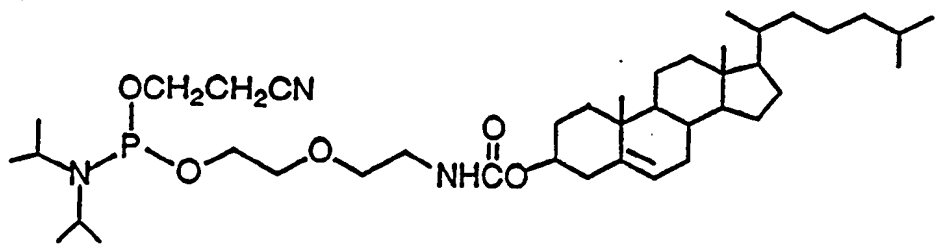

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FIGURE 3

Scheme 4: Synthesis of thiocholesterol-modified oligonucleotide

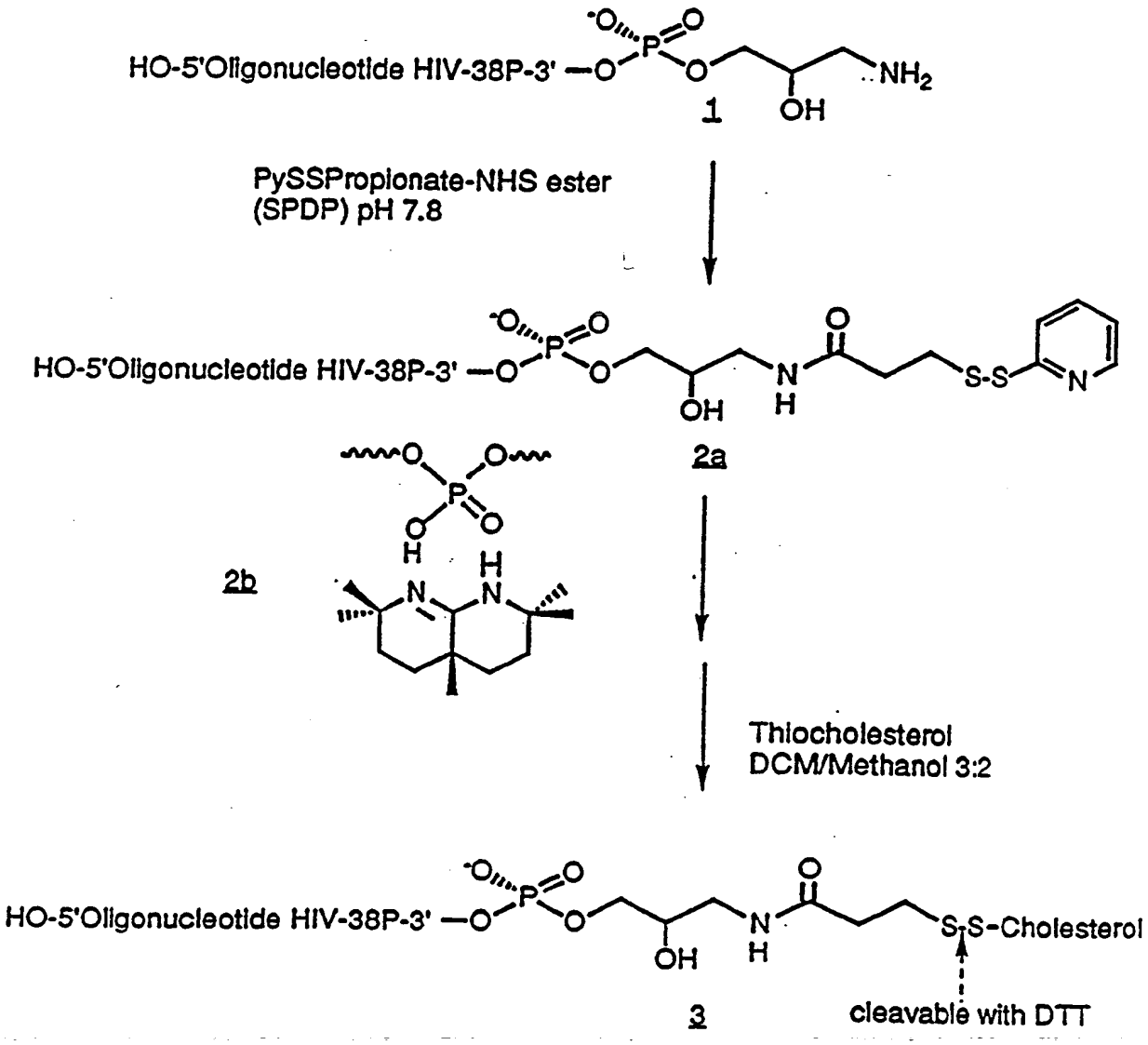


FIGURE 4

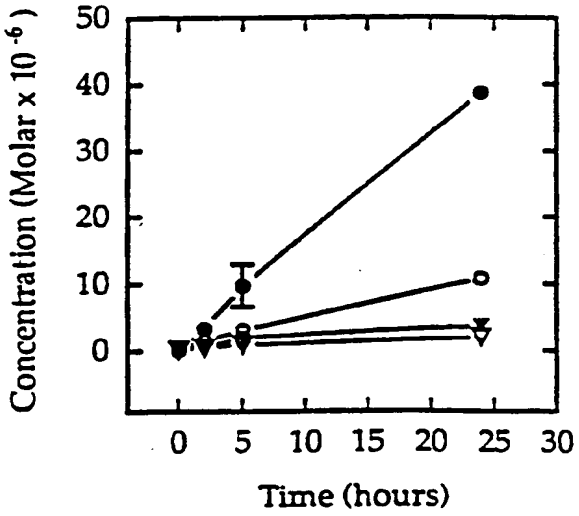


FIGURE 5

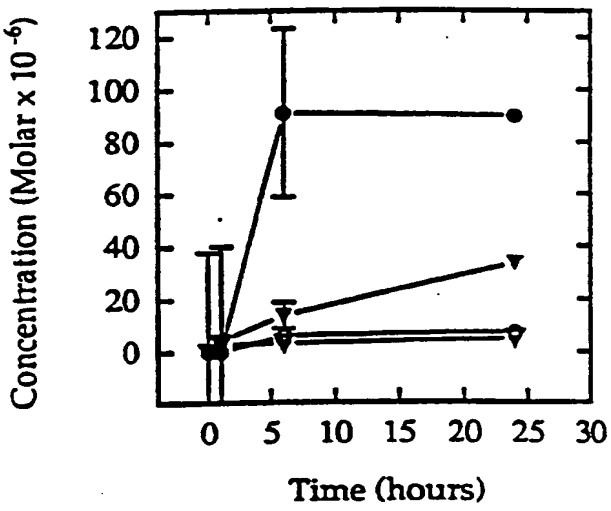
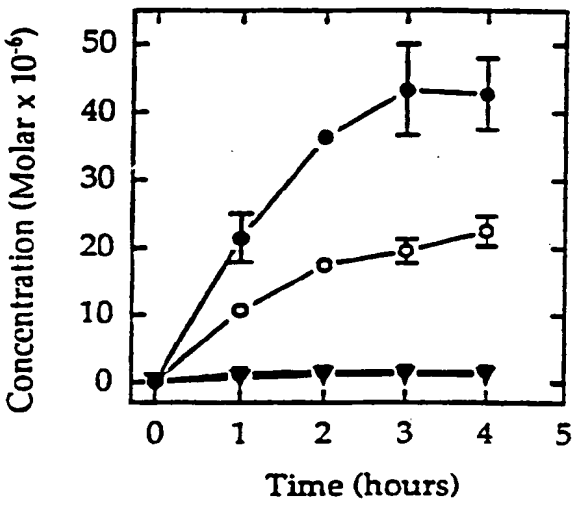
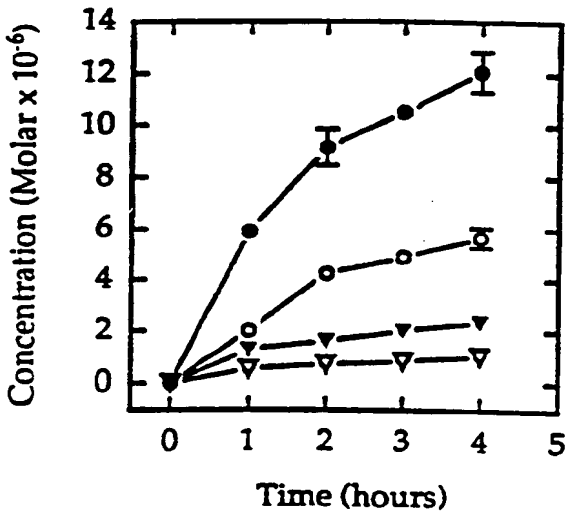


FIGURE 6



7 / 19 FIGURE 7

SUBSTITUTE SHEET



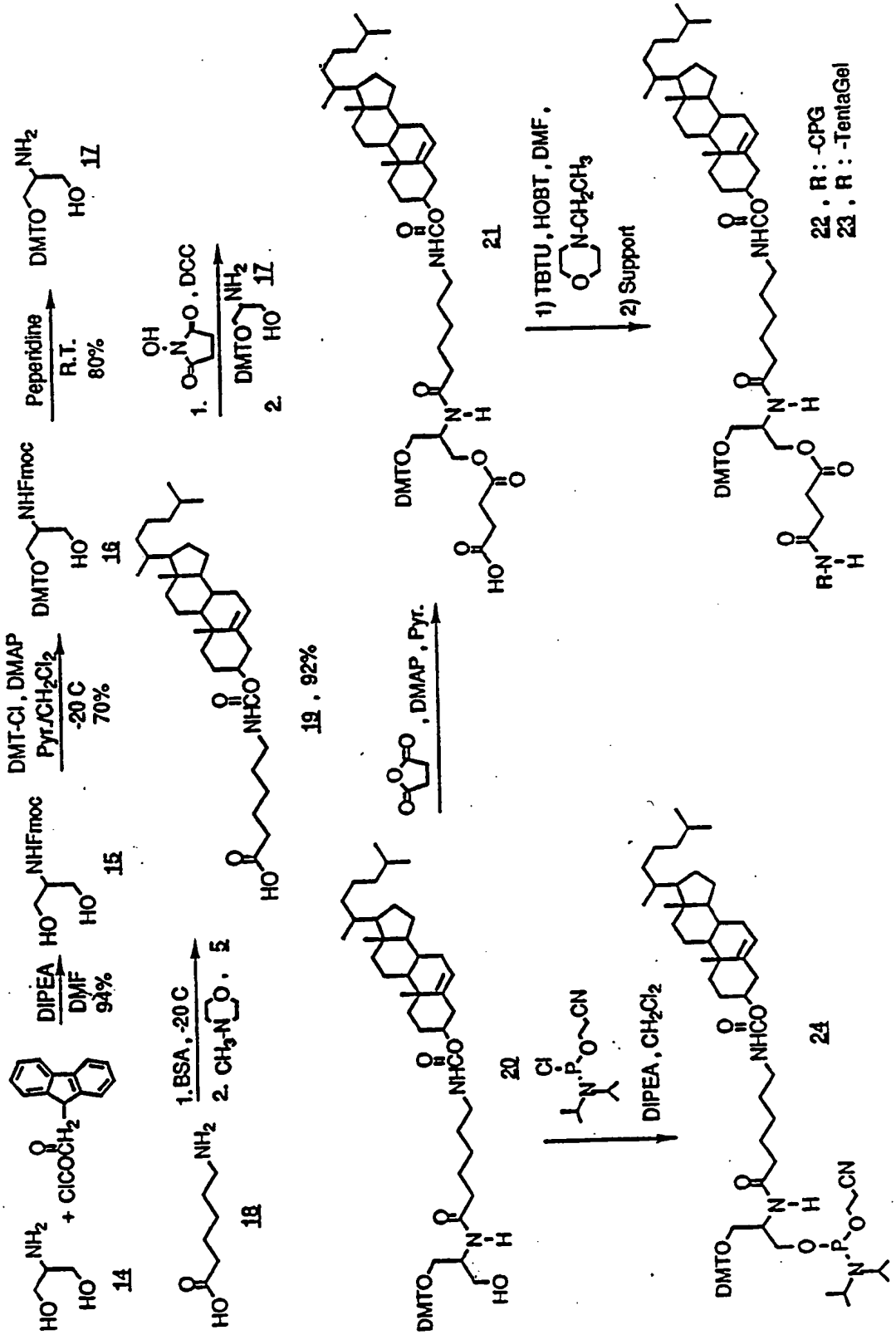


FIGURE 9

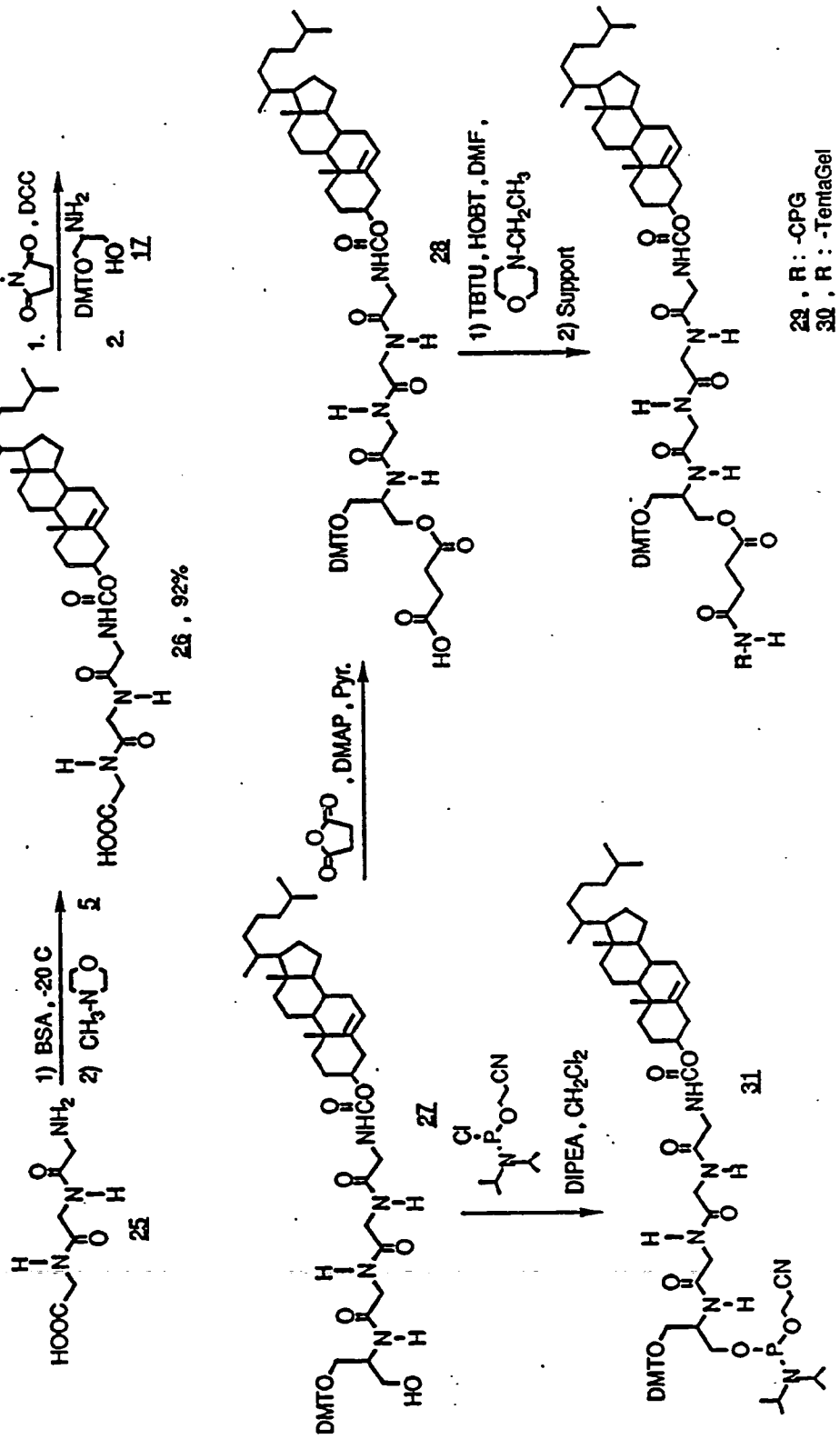


FIGURE 10

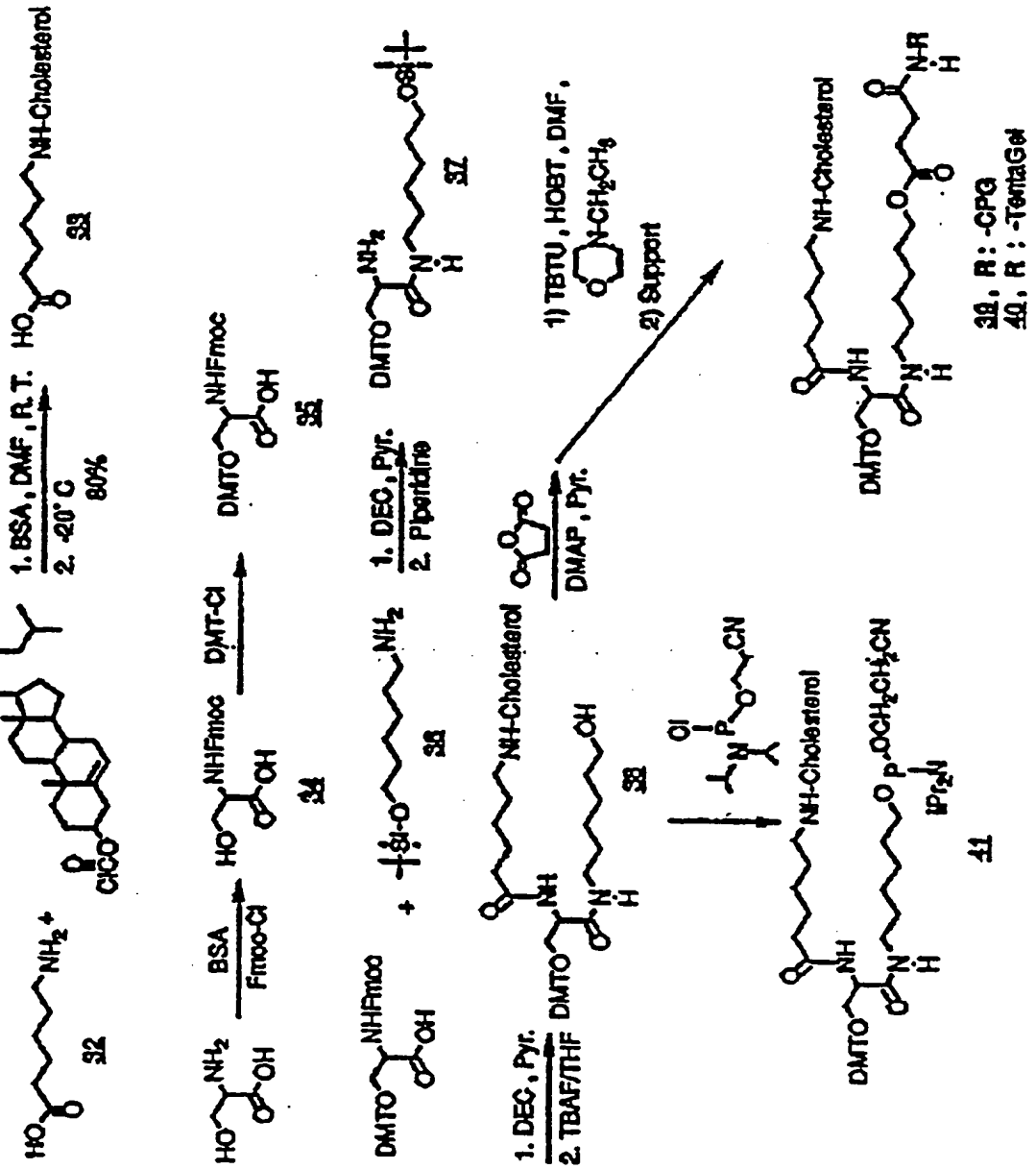


FIGURE 11

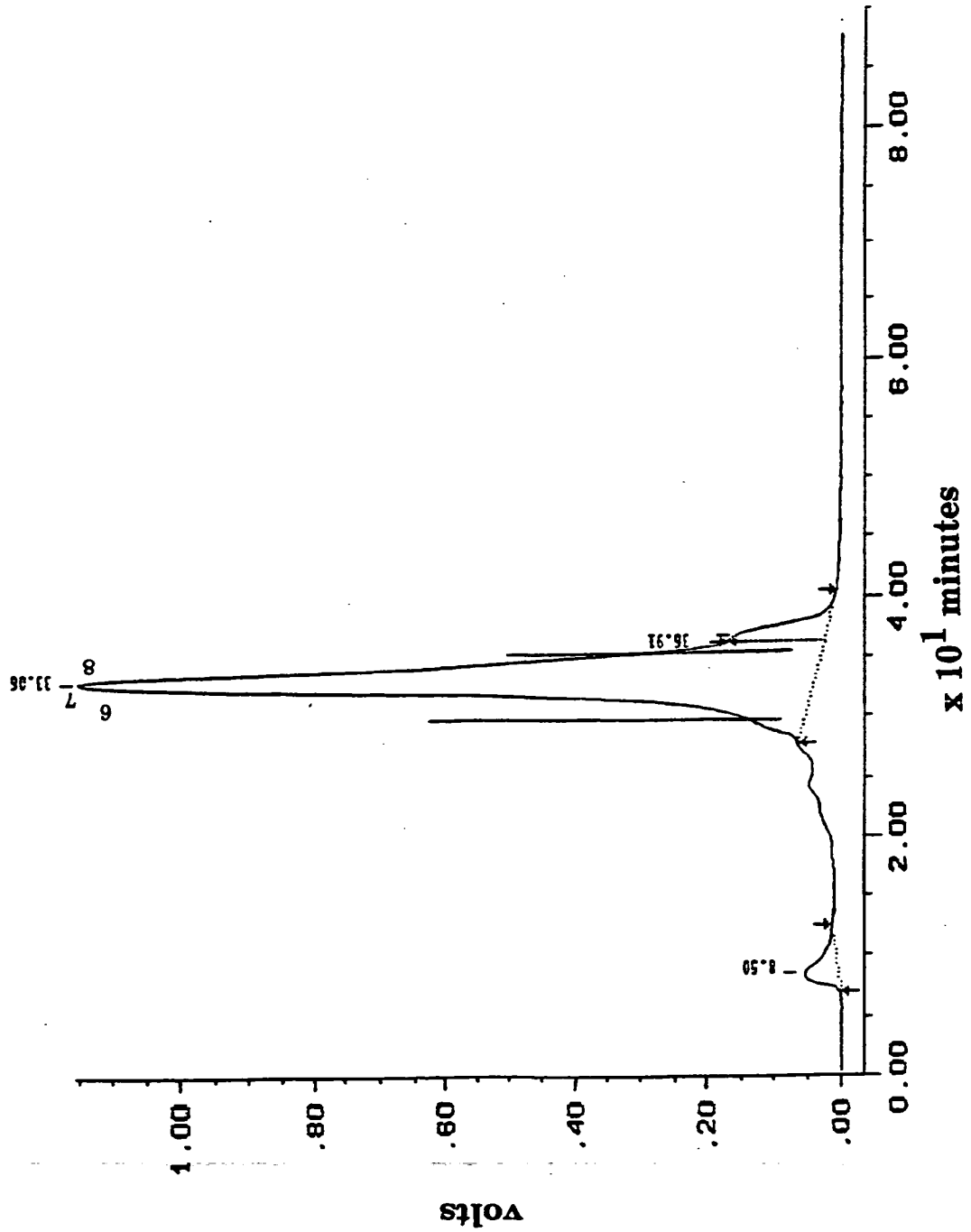


FIGURE 12

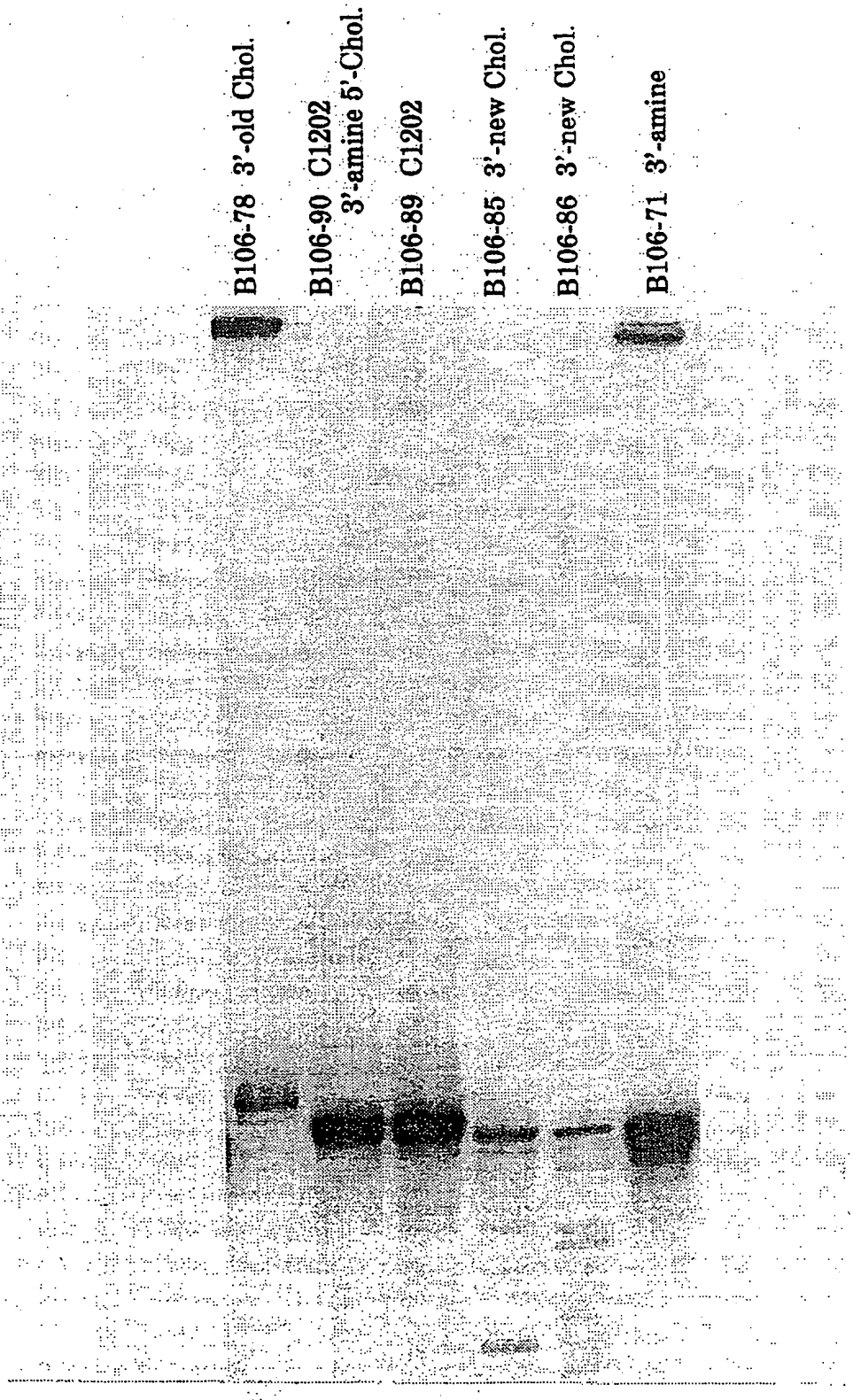


FIGURE 13

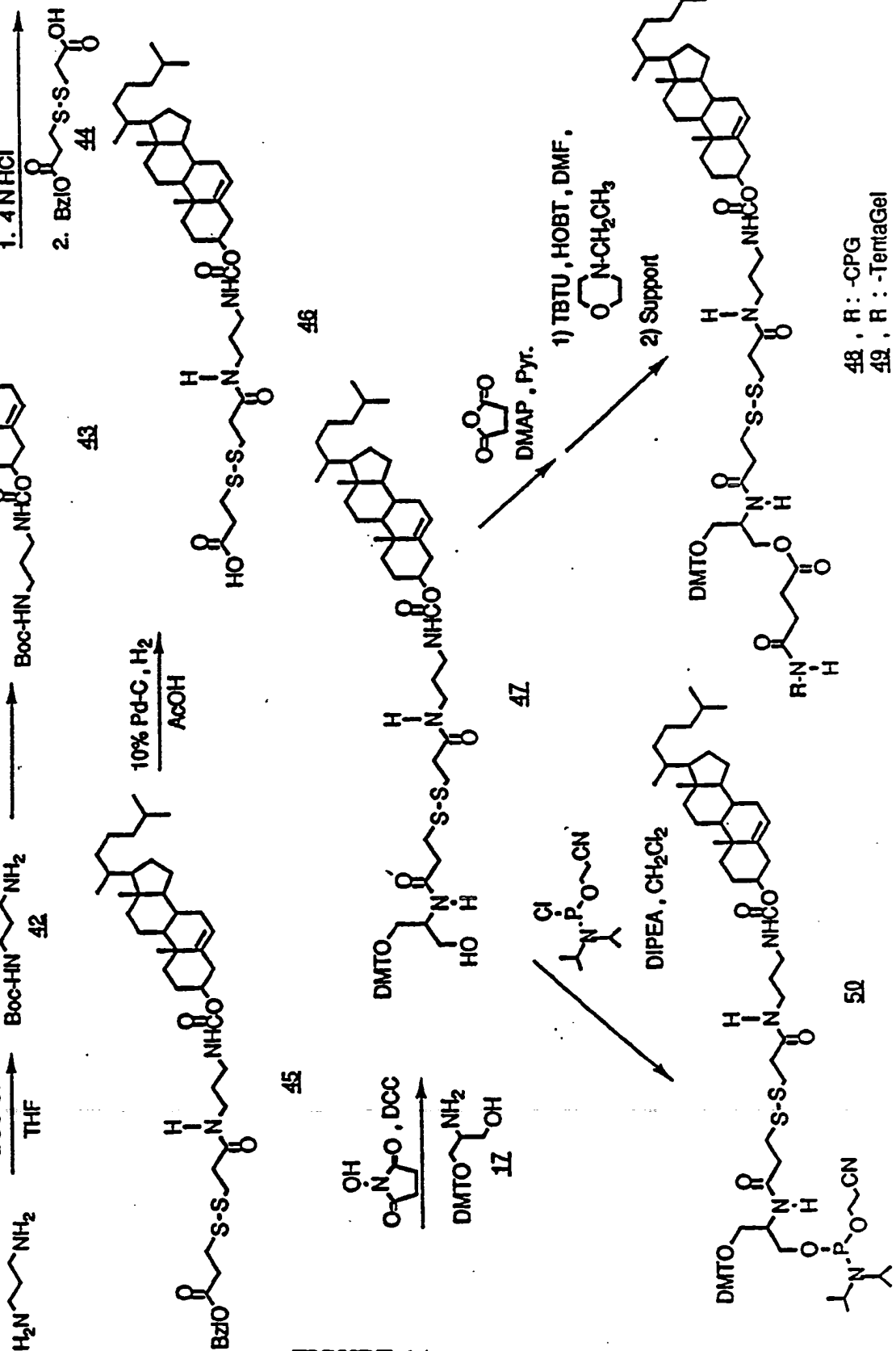
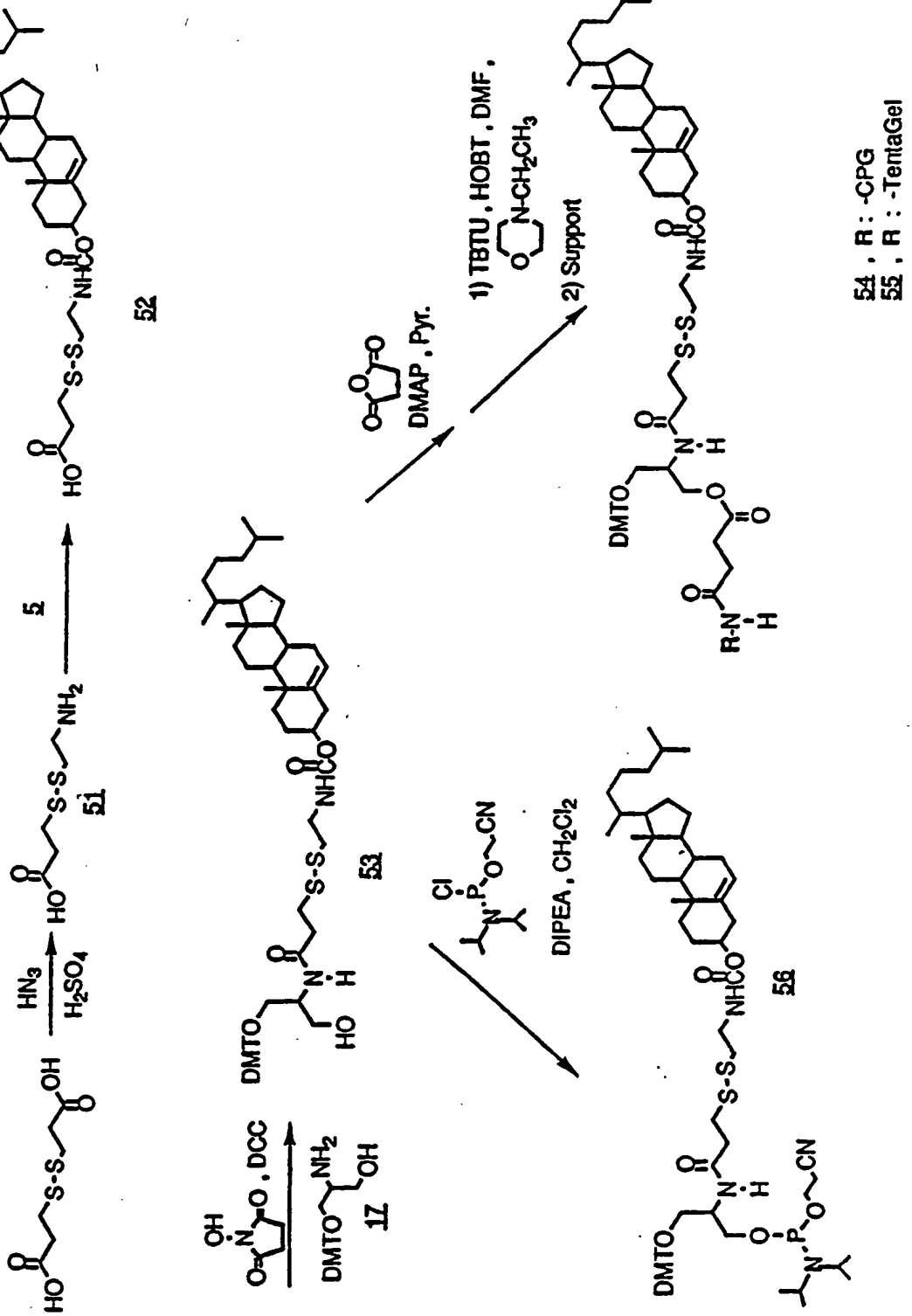


FIGURE 14



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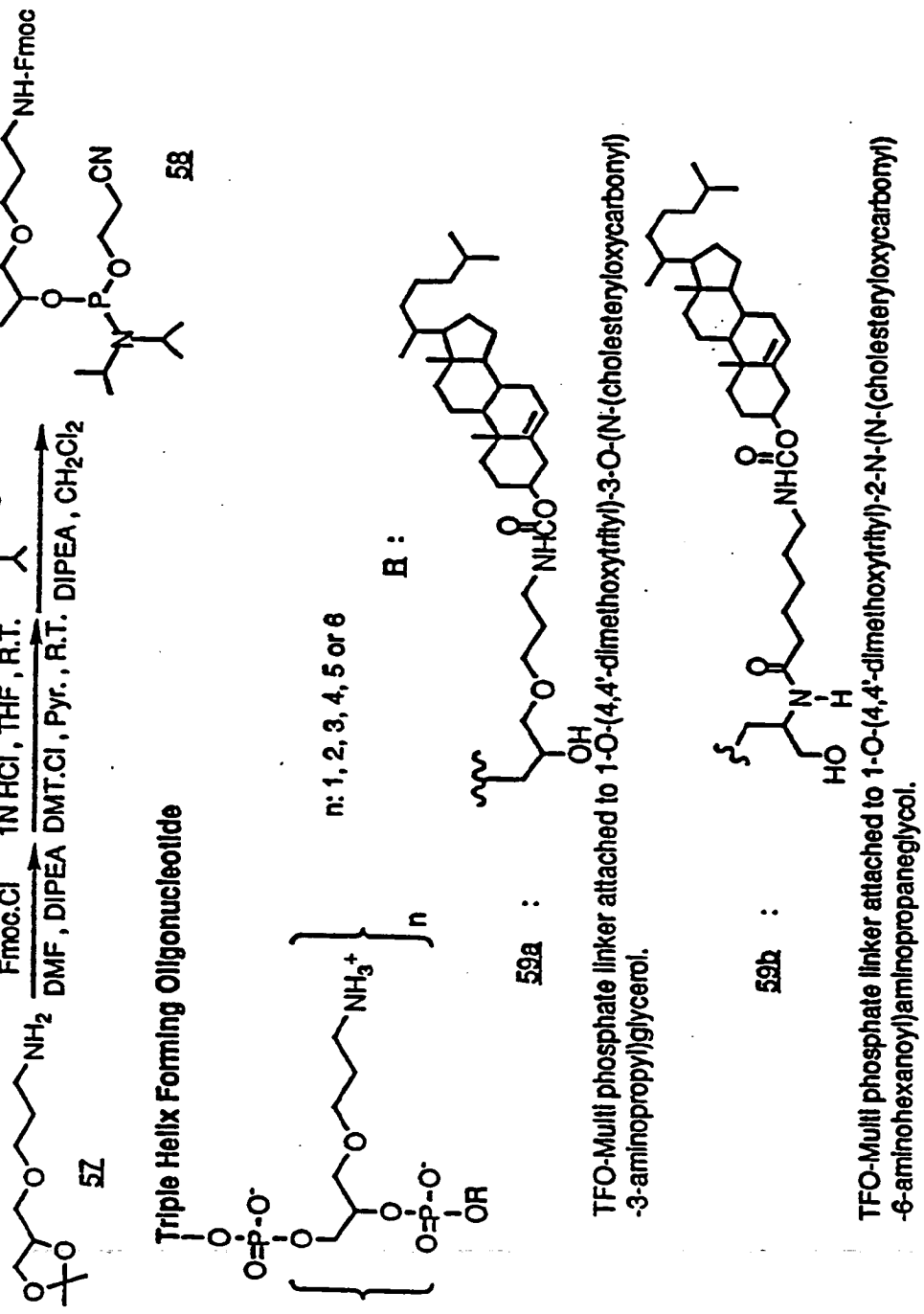
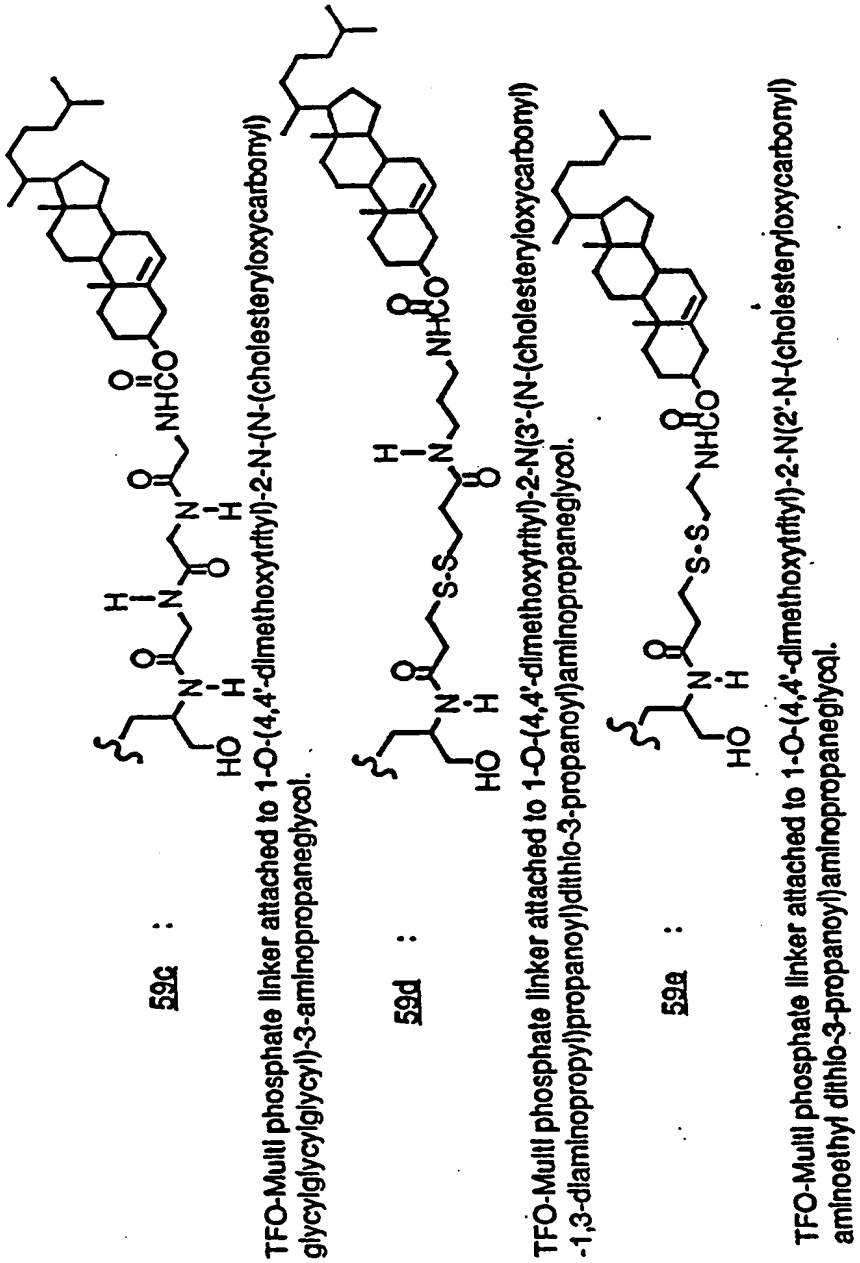


FIGURE 16



59c : TFO-Multi phosphate linker attached to 1-O-(4,4'-dimethoxytrityl)-2-N-(N-(cholesteryloxycarbonyl) glycylglycylglycyl)-3-aminopropaneglycol.

59d : TFO-Multi phosphate linker attached to 1-O-(4,4'-dimethoxytrityl)-2-N(3'-(N-(cholesteryloxycarbonyl) -1,3-diaminopropyl)propanoyl)dithio-3-propanoyl)aminopropaneglycol.

59e : TFO-Multi phosphate linker attached to 1-O-(4,4'-dimethoxytrityl)-2-N(2'-N-(cholesteryloxycarbonyl) aminoethyl dithio-3-propanoyl)aminopropaneglycol.

FIGURE 16

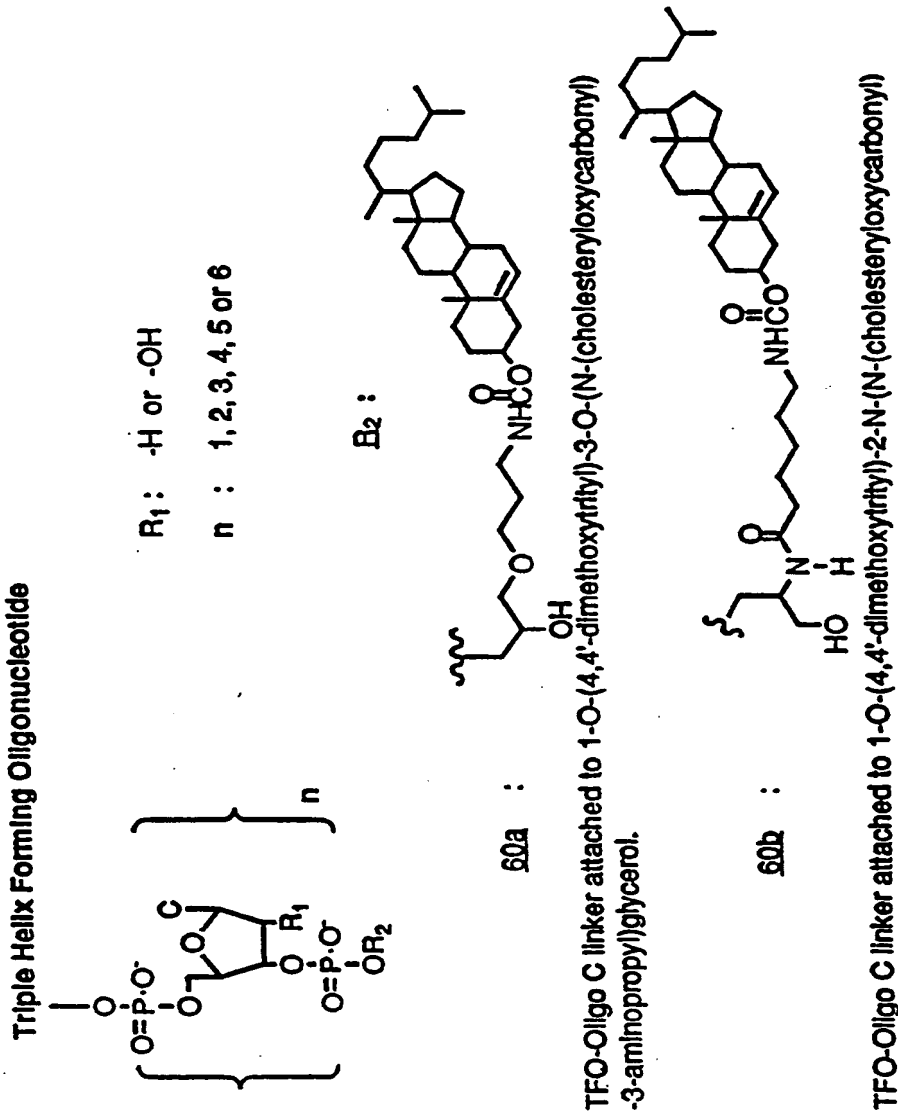


FIGURE 17

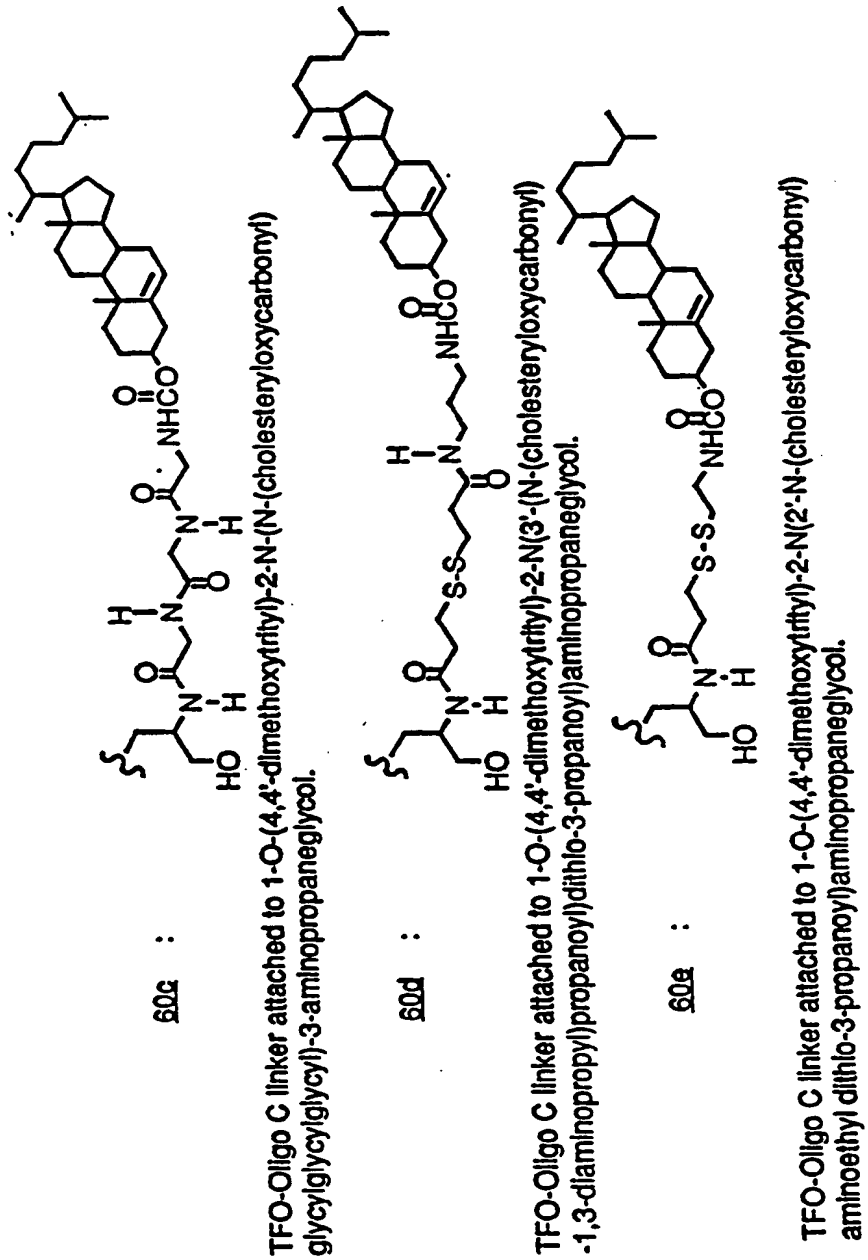


FIGURE 17

INTERNATIONAL SEARCH REPORT

Int. ional application No.
PCT/US93/07743

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07H 21/02; A61K 48/00; C12Q 1/68
US CL :536/22.1; 435/6; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/22.1; 435/6; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GenBank, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nucleic Acids Research, Vol. 20, No. 3, issued 1992, Oberhauser et al., "Effective incorporation of 2'-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol", pages 533-538, see entire document.	1-28
A	Nucleic Acids Research, Vol. 18, No. 13, issued 1990, Shea et al, "Synthesis, hybridization properties and antiviral activity of lipid-oligodeoxynucleotide conjugates", pages 3777-3783, see entire document.	1-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 NOVEMBER 1993	Date of mailing of the international search report 03 DEC 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer MINDY B. FLEISHER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★